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GE11 positive exosomes as a potential RNAi delivery system in clear cell renal cell carcinoma

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Abbreviations

A

AKT Protein kinase B

AMOs anti-miRNA oligonucleotides

B

BACE Beta-secretase

BMDC Bone-marrow derived cells

C

CAF Cancer-associated fibroblast

CBP cAMP response elements-binding protein

ccRCC Clear cell renal cell carcinoma

cDNA Complementary deoxyribonucleic acid

CXCR4 Chemokine (C-X-C motif) receptor 4

D

DC Dendritic cell

DNA Deoxyribonucleic acid

E

EAU European Association of Urology

ECM Extracellular matrix

ECV Elongins B and C/Cullin 2/pVHL

EGF Epidermal growth factor

EGFR Epidermal growth factor receptor

EPO Erythropoietin

ERK Extracellular-signal-regulated kinases

EV Extracellular vesicle

F

FBS Fetal bovine serum

FLOT1 Flotillin 1

G

GLUT1 Glucose transporter 1

GPC1 Glypican 1

GRB2 Growth factor receptor-bound protein 2

H

HA Hemagglutinin

HIF Hypoxia inducible factor

HIFα Hypoxia inducible factor alpha

HIFβ Hypoxia inducible factor beta

HRE Hypoxia-responsive elements

HRP horseradish peroxidase

I

IARC International Agency for Research on Cancer

IL-2 Interleukin 2

IMC Immature myeloid cell

INFα Interferon alpha

ITS Insuline-transferrine-selenium

L

LDHA Lactose desydrogenase A

LNA Locked Nucleic Acid

M

MAPK Mitogen-activated protein kinases

MCL Mantle cell lymphoma

miRNA microRNA

mRCC metastatic renal cell carcinoma

mRNA messenger ribonucleic acid

MSC Mesenchymal stem cell

mTOR mammilian target of rapamycin

MVB Multivesicular bodies

P

PDGFβ	Platelet derived growth factor Beta
PDGFR	Platelet derived growth factor receptor
PDK	Phosphoinositide-dependent kinase
PFA	Paraformaldehyde
PGK	Phosphoglycerate kinase
PI3K	Phosphatidylinositol 3 kinase
PLCγ	Phosphoinositide phospholipase C gama
PVDF	Polyvinylidene Difluoride
pVHL	Protein von Hippel-Lindau

R

RCC	Renal cell carcinoma
RISC	Ribonucleic acid -induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RVG	Rabies virus glycoprotein

S

SDS	Sodium dodecylsulphate
SEM	Standard error of the median
siRNA	small interfering ribonucleic acid
STAT	Signal transducers and activators of transcription

T

TGFα	Transforming growth factor alpha
TME	Tumor microenvironment
TKI	Tyrosine kinase inhibitor
TKR	Tyrosine kinase receptor

U

UTR	Untranslated region

V

VEGF Vascular endothelial growth factor

VEGFR Vascular endothelial growth factor receptor

VHL von Hippel-Lindau

W

WHO World Health Organization

Resumo

O carcinoma de células renais é a neoplasia renal mais comum e compreende três principais subtipos histológicos, sendo o carcinoma de células renais de células claras (CCRcc) o mais prevalente e agressivo. O CCRcc é frequentemente caraterizado pela perda, mutação ou silenciamento epigenético do gene supressor tumoral *Von-Hippel Lindau* (*VHL*). A inativação do *VHL* e consequente acumulação do fator induzido pela hipoxia (HIF) leva à ativação de vias de sinalização associadas a um contexto de hipoxia. Nomeadamente, a produção do fator de transformação α (TGFα) conduz à ativação do fator de crescimento epidérmico (EGFR). Adicionalmente, estudos demonstraram a sobreexpressão do EGFR e o aumento da sua estabilidade na membrana de células de CCRcc. Os doentes de CCRcc desenvolvem resistência às terapias aplicadas na clínica, pelo que existe a necessidade de desenvolver novas estratégias terapêuticas que ultrapassem estas limitações. A desregulação do EGFR pode ser a base para o desenvolvimento de novas terapias dirigidas. Recentemente, os exossomas têm surgido como novas ferramentas terapêuticas dada a possibilidade de serem modificados e escaperem ao sistema imune. Estas vesículas extracelulares de 30 a 150 nm podem mediar a comunicação intercelular através do transporte de moléculas bioativas (proteínas, *DNA* e microRNAs (miRNAs)). O GE11 é um péptido que se liga especificamente ao EGFR e pode ser expresso na membrana dos exossomas, permitindo especificidade de ligação. Em última instância, estas vesículas extracelulares podem ser carregadas com RNA de interferência (RNAi), pequenas moléculas de RNA que controlam a expressão genética a nível pós-transcricional. Estudos recentes demonstraram o envolvimento de miRNAs na regulação do eixo VHL/HIF, e consequentemente das vias de sinalização relacionadas com o EGFR. Os miRNAs são pequenos RNAs não codificantes responsáveis pela regulação de inúmeros genes a nível pós-transcricional. Estudos bioinformáticos revelam que a p53 pode ser um dos alvos do miRNA-1233. A p53 regula os níveis de HIF através da degradação mediada pelo MDM2. Desta forma, o miRNA-1233 é um candidato promissor a alvo terapêutico visto que poderá regular os níveis da p53 e consequentes respostas de HIF relacionadas com a hipoxia. Portanto, o objetivo deste estudo é avaliar o uso de exossomas positivos para o GE11 ($GE11^{+}$) como um veículo de entrega para o potencial uso de RNAi em CCRcc. No presente estudo, foi usada uma linha celular caraterizada como metastática de CCRcc, FG-2, duas linhas celulares epiteliais do túbulo proximal de rim, HKC-8 e HK-2 e uma linha celular embrionária de rim, HEK293T. De acordo com os resultados obtidos, a linha celular FG-2 apresentava níveis de expressão do mRNA do *EGFR* ($P<0.001$) e níveis proteicos do recetor superiores comparativamente à linha celular HKC-8. A via de sinalização

MAPK/ERK encontrava-se sobreativa uma vez que a linha celular FG-2 apresentava níveis superiores da forma ERK2 fosforilada comparativamente à linha celular HKC-8. Estes resultados realçam a importância do EGFR no desenvolvimento de CCRcc e o potencial uso de um sistema de entrega ao EGFR baseado em exossomas. A manipulação da linha celular HEK293T permitiu a obtenção de exossomas com o péptido GE11 na membrana. Estes exossomas foram marcados fluorescentemente e incubados com as linhas celulares HK-2 ou FG-2 a 4°C ou 37°C durante 4 horas. A 4°C observou-se uma quase completa inexistência de internalização dos exossomas em ambas as linhas celulares, sugerindo que as células devem estar biologicamente ativas para que ocorra a internalização de exossomas. Relativamente à incubação a 37°C, uma percentagem mais elevada de células FG-2 internalizou os exossomas $GE11^+$ comparativamente aos exossomas derivados de HEK293T transfetadas com pDisplay, a condição controlo (97% e 92% respetivamente, $P<0.001$). Adicionalmente, observou-se que as células FG-2 que internalizaram os exossomas $GE11^+$ apresentavam uma mediana de intensidade de fluorescência 1.4 vezes superior comparativamente ao controlo ($P=0.001$), sugerindo que cada célula terá internalizado um maior número de exossomas através de um mecanismo dependente de EGFR. Relativamente à linha celular HK-2, não foram observadas diferenças de internalização de exossomas $GE11^+$ e o controlo, visto que os níveis proteicos do EGFR eram inferiores comparativamente à linha celular FG-2. Em suma, foi possível observar uma diferença estatisticamente significativa dos níveis de internalização dos exossomas $GE11^+$ entre as linhas celulares normal e tumoral (22% e 97%, respetivamente, $P=0.001$). Contudo, estas diferenças de internalização não se deveram exclusivamente aos diferentes níveis de expressão do EGFR entre as linhas renais, uma vez que se observou uma diferença entre os níveis de internalização na condição controlo (19% e 92%, respetivamente, $P<0.001$). Em conclusão, o uso de exossomas $GE11^+$ pode constituir um novo sistema de entrega de RNAi em CCRcc. Estudos iniciais demonstraram que o miRNA-1233 e o mRNA da *TP53* se encontravam respetivamente sobreexpresso ($P=0.007$) e subexpresso ($P=0.016$), na linha celular FG-2 comparativamente à linha celular HKC-8. Estudos adicionais realizados na linha celular FG-2 demonstraram que 24 ou 48 horas após a transfeção com um mímico do miRNA-1233, os níveis de expressão do mRNA da *TP53* não se alteraram. Contudo, o miRNA-1233 pode atuar ao nível do mRNA da *TP53*, inibindo a tradução e levando à diminuição dos níveis proteicos da p53 em vez de levar à degradação do mRNA. Todavia, o miRNA-1233 poderá constituir um promissor alvo terapêutico dado o seu papel oncogénico em CCRcc. O uso de exossomas $GE11^+$ carregados com um antagonista do miRNA-1233 poderá afetar o desenvolvimento tumoral de CCRcc, tornando-se numa nova abordagem terapêutica.

Abstract

Renal cell carcinoma is the most common solid kidney cancer and comprises three major histological subtypes, being the clear cell Renal Cell Carcinoma (ccRCC) the most prevelant and aggressive one. ccRCC is frequently characterized by the loss, mutation or epigenetic silencing of the *Von-Hippel Lindau* tumor suppressor gene (*VHL*). *VHL* impairment and consequent Hypoxia Inducible Factor (HIF) accumulation induces a state of hypoxia in non-hypoxic conditions, responsible for the dysregulation of several pathways, including the epidermal growth factor receptor (EGFR) activation through the production of transforming growth factor α (TGFα). Additionally, studies demonstrated EGFR overexpression and an increase in the receptor's stability in the cell membrane in ccRCC. Since ccRCC patients end up developing resistance to the therapies applied in the clinic, there is the need to develop new therapeutic strategies that overcome these therapy limitations. EGFR dysregulation could be the base for the development of new targeted therapies. Recently, the exosomes have emerged as promising therapeutic tools due to their engineering potential and ability to evade the immune system. These extracellular vesicles of 30 to 150 nm can mediate intercellular communication through the shuttle of bioactive molecules like proteins, DNA, and microRNAs (miRNAs). GE11 is a synthetic peptide that specifically binds EGFR and can be expressed in the membrane of exosomes, allowing a specific delivery. Ultimately, these extracellular vesicles can be loaded with RNA interference (RNAi)-based drugs, small interfering RNA molecules that control gene expression post-transcriptionally, allowing a more specific deliver and potent drug effect. Increasing evidence supports the involvement of miRNAs in VHL/HIF axis regulation, ultimately affecting EGFR-related pathways. MiRNAs are small non-coding RNAs, responsible for numerous genes regulation at a post-transcriptional level. Studies proven that HIF can be directly regulated by p53 through MDM2-mediated degradation. *In silico* approaches predicted p53 as a miRNA-1233 target. Thus, miRNA-1233 could regulate p53 levels and consequent HIF responses-related to hypoxia, such as EGFR activation making him a promising therapeutic target in ccRCC. Therefore, the aim of this study is to evaluate the potential use of GE11-positive ($GE11^+$) exosomes as a delivery vehicle for RNAi-based therapy in cancer cells overexpressing EGFR, using ccRCC as a model. In the present study, *in vitro* experiments were performed using a metastatic ccRCC cell line, FG-2, two normal proximal tubule epithelial renal cell lines, HKC-8 and HK-2 and a human embryonic kidney-derived cell line, HEK293T. According to our results, *EGFR* mRNA expression levels were higher in FG-2 cells when compared to HKC-8 cells ($P<0.001$). Moreover, tumor cells also presented higher protein levels of EGFR as well as higher phosphorylated levels

when compared to normal cells. In addition, downstream signaling MAPK/ERK was overactivated since higher levels of ERK2 phosphorylated form were found in FG-2 cells comparing to HKC-8 cells. Taken together, these results highlight the importance of EGFR in ccRCC development and the potential use of an EGFR-based exosomes delivery system. Exosomes derived from HEK293T were engineered in order to express the GE11 peptide on their surface, labeled with a fluorescent dye and incubated with either HK-2 or FG-2 cell lines at 4°C or 37°C for 4 hours. After incubation at 4°C there was almost complete abrogation of exosomes internalization by both renal cell lines, suggesting that cells had to be biologically active in order for this process to occur. Regarding cells incubation with exosomes at 37°C, a higher percentage of FG-2 cells internalized $GE11^+$ exosomes comparing to exosomes derived from HEK293T cells transfected with pDisplay, the control condition (97% and 92%, respectively, $P<0.001$). In addition, by analyzing the fluorescence intensity from FG-2 cells we observed that cells that internalized $GE11^+$ exosomes had a 1.4 greater fluorescence intensity median comparing to control ($P=0.001$), suggesting that each cell uptakes more $GE11^+$ exosomes, in an EGFR-dependent manner. Concerning HK-2 cells, differences between the internalization of $GE11^+$ exosomes and the control were not observed since their protein EGFR levels were lower when compared to FG-2 cells. Overall, there was a statistical significant difference between $GE11^+$ exosomes uptake by the normal and tumor renal cell lines (22% and 97%, respectively, $P=0.001$). However, uptake differences of $GE11^+$ exosomes were not exclusively due to different EGFR expression levels between normal and tumor renal cell lines, since there was a significant difference concerning the uptake levels in the control condition (19% and 92%, respectively, $P<0.001$). Overall, the use of $GE11^+$ exosomes is a very promising new delivery system in ccRCC and probably in others EGFR-overexpressing tumors. Initial findings demonstrated that miRNA-1233 was upregulated ($P=0.007$) whereas, *TP53* mRNA was downregulated ($P=0.016$) in FG-2 cells comparing to HKC-8 cells. Additional studies performed in FG-2 cells showed that 24 or 48 hours after transfection with a miRNA-1233 mimic, *TP53* mRNA expression levels did not change. However, miRNA-1233 can still target *TP53* mRNA and lead to its protein levels downregulation through translation inhibition, instead of mRNA degradation. Nonetheless, miRNA-1233 dysregulation and its potential oncogenic role in ccRCC makes it a new promising candidate for drug targeting. The use of $GE11^+$ exosomes loaded with a miRNA-1233 antagonist could potentially affect ccRCC tumor development, becoming a novel therapeutic approach.

1. Introduction

1.1 Molecular biology of cancer

Cancer is an emergent public health issue. It is one of the leading causes of death worldwide [1]. According to GLOBOCAN 2012, the International Agency for Research on Cancer (IARC) online database, in 2012 there were an estimated 14.1 million new cases of cancer diagnosed worldwide (excluding non-melanoma skin cancer) and 8.2 million cancer-related deaths [2]. Over the past years, cancer burden has increased in economically developing countries as a result of population aging and growth. Additionally, certain lifestyles and cancer-promoting behaviors such as smoking, physical inactivity, and 'westernized' diets have also contributed to the ascending numbers [3]. Thus, by the year of 2030, GLOBOCAN estimates an astonishing increase of approximately 77% of new cancer cases per year and 13 million deaths worldwide [4].

Cancer development is a multifactorial and multistep process which includes the following stages: initiation, promotion and progression [5]. Cells are continuously exposed to different types of aggressions that ultimately, upon failure of cell repairing machinery, can lead to a genetic and/or epigenetic modification in the DNA. On the one hand, genetic alterations can include chromosomal rearrangements and translocations, deletions, insertions, amplification of certain genes and point mutations. Most of the times, these genetic alterations occur in the somatic cells of the individual, whereas cancer predisposition syndromes with inherited mutations remain rare events [6]. On the other hand, epigenetic changes are often related with focal increases and/or decreases in DNA methylation and alterations in histone post-translational modifications [7]. Altered genes can be categorized in proto-oncogenes or tumor suppressor genes regarding their role in tumor initiation and development. The proto-oncogenes encode proteins that regulate normal cellular growth and differentiation, while mutated versions of these genes are named oncogenes and only require mutation in one allele to act dominantly and promote carcinogenesis. The tumor suppressor genes encode proteins that normally suppress cellular proliferation and both alleles must be mutated to interfere with normal cell function [8, 9]. Moreover, additional mutations in genes involved in DNA repair, cell-cycle control, angiogenesis and telomerase production are also needed [6, 8]. Overall, these alterations contribute to the transformation of a normal cell into neoplastic. Proteins affected

by these genetic alterations include growth factors and their receptors, kinase inhibitors, signal transducers and transcription factors. Subsequently, multiple events occur that result in the disruption of key cellular processes such as cell proliferation and apoptosis, which in turn leads to tumor growth [6]. In fact, cells become less sensitive to apoptotic signals, cell growth arrest and destruction by the immune system. Furthermore, the self-sufficiency of these cells in growth factors provides them with greater proliferative capacity, angiogenesis and invasive potential. These characteristics combined with increased inflammation, genomic instability and metabolism dysregulation, are considered the *Hallmarks of Cancer* and, ultimately, favor tumor development, invasion and systemic dissemination [10, 11].

However, cancer is no longer perceived as a cellular disease defined exclusively by genomic alterations. Nowadays, cancer is seen as an ecological disease involving a dynamic and complex interplay between malignant and non-malignant cells [12, 13]. This new approach emphasizes the role of tumor microenvironment (TME) in cancer niche formation and its importance through tumor initiation and development processes [14]. Recently, Barcellos-Hoff and colleagues proposed the division of cancer niche evolution into three phases: construction, expansion and maturation (Figure 1) [13].

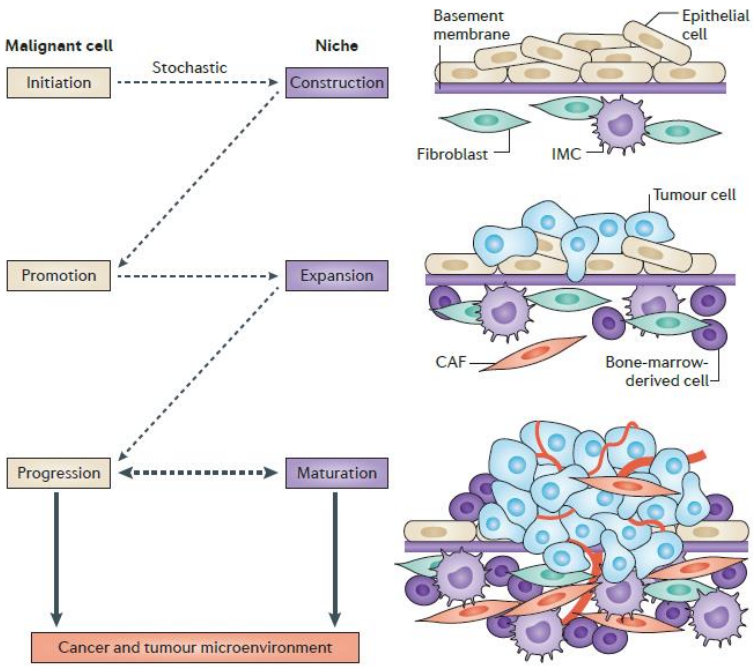

Figure 1 – Diagram elucidating the interdependent processes that can occur in cancer niche evolution (niche construction, expansion and maturation) in parallel with classical multistep carcinogenesis model (initiation, promotion and progression). During niche construction stromal cells contribute to the survival of transformed cells. Niche expansion supports tumor cell diversity and contributes to angiogenesis, and eventually matures into the tumor microenvironment. CAF, cancer-associated fibroblast; IMC, immature myeloid cell. Adapted from Barcellos-Hoff *et al.* [13].

Briefly, niche construction consists in the interaction between activated stromal cells and normal cells, enabling transformed clone survival. Consequently, niche expansion generates secreted factors such as chemokines, cytokines, exosomes and other molecules that remodel local tissue concomitant with initiated clone expansion. In fact, one of the predominant methods of communication between cells is through the exchange of bioactive molecules like proteins and microRNAs that are transported inside of exosomes, defined as extracellular vesicles (EV). Then, the recruitment of both bone-marrow-derived cells (BMDCs) and resident cells (especially fibroblasts) drives niche maturation up to an established TME [13]. The TME is composed by numerous cell types that surround tumor cells and include cells of the immune system such as lymphocytes, macrophages, natural killer cells and others, endothelial cells, fibroblasts, mesenchymal stem cells (MSCs) and the extracellular matrix (ECM). Each of these components interacts with and influences malignant cells, ultimately promoting tumor development [15]. Overall, these considerations highlight the complexity behind tumor initiation and progression that comprise a multifactorial communication between different cell types.

1.2 Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the most common solid kidney cancer, accounting for 2-3% of all adult malignancies and 90% of all kidney neoplasms [16]. Considered the most lethal urologic tumor, the RCC is responsible for more than 100 000 deaths worldwide each year, and with incidence and mortality rates increasing approximately 2% per decade [17, 18].

Incidence and mortality rates of RCC also reflect a geographic variation (Figure 2). The highest incidence rates are observed in Northern America, Western Europe and Australia/New Zealand, whereas relatively low rates are observed in Africa and South-East Asia. The highest mortality rates are registered in Europe, mainly in the central and eastern countries, followed by the western and southern regions [19].

The geographic disparity observed in RCC incidence rates worldwide can be attributed to differences in frequency of diagnostic imaging, access to health care, genetic inheritance and prevalence of certain lifestyle habits and/or environmental risk factors [20].

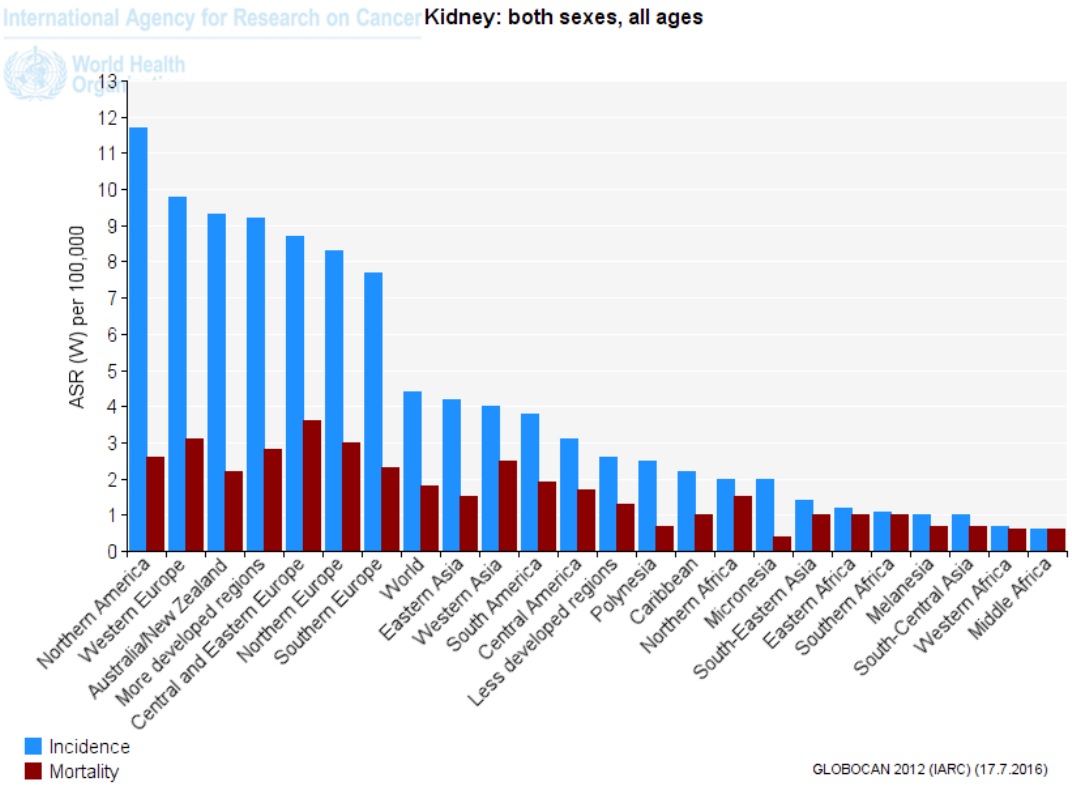

Figure 2 – Distribution of incidence and mortality rates of kidney cancer by 100 000 habitants (GLOBOCAN 2012, IARC [4]).

RCC etiology has not yet been completely elucidated. However, epidemiologic studies revealed an association between certain lifestyles and disease development. In accordance with the European Association of Urology (EAU) there is a 3:2 predominance of new cases diagnosed in men over women, with peak incidence occurring between the age of 60 and 70 years old [21]. Established risk factors for RCC include smoking, obesity, hypertension, acquired cystic disease, family history of RCC, and exposure to various chemical compounds [22, 23]. In addition, alcohol consumption, diabetes and nutritional factors also seem to modulate RCC development but further studies are needed in order to clarify their association with the disease [20, 24].

Currently, more than 50% of RCCs are detected incidentally as a consequence of non-invasive imaging examinations, such as computed tomography and magnetic resonance imaging, used to investigate non-specific symptoms and other abdominal diseases [21, 25]. This fact can be explained since the majority of renal masses remain asymptomatic until late stages of the disease [21]. Therefore, one-third of the patients are initially diagnosed with locally invasive or metastatic disease [25]. For patients diagnosed with localized RCC surgical intervention is the primary treatment choice [26]. However, despite being considered curative, recurrence still occurs in 20-40% of patients depending on the tumor stage and grade [27]. Moreover, surgery

alone has limited benefit in patients with metastatic disease, except for palliative reasons [18, 26]. Until the past decade, treatment options for patients with metastatic RCC (mRCC) have been extremely limited since RCC is notoriously resistant to chemotherapy and radiotherapy [28, 29]. Prior to the use of anti-angiogenic target agents, systemic treatment options were limited to cytokine therapies (interleukin – IL-2 and interferon α– IFNα). However, these approaches have limited therapeutic benefits [30, 31]. In the past years, following the identification of various molecular pathways linked to RCC initiation and progression, targeted agents such as receptor tyrosine kinase inhibitors (TKI), vascular endothelial growth factor (VEGF) antibodies, and mammalian target of rapamycin inhibitors (mTORs) are now a crucial part of most therapeutic strategies for patients with mRCC [25, 26, 32]. Although mRCC patient's outcome has improved, tumor cells eventually become resistant to the targeted therapies applied due to genetic and epigenetic changes but mainly through downstream activation of the signaling pathway targeted therapy site [33-35].

1.3 Molecular biology of clear cell Renal Cell Carcinoma

RCC is not a single-entity disease. Instead, consists of different morphologic subtypes with distinct genetic backgrounds [36]. According to the ISUP Vancouver Modification of WHO there are more than twenty-four RCC subtypes, being the clear cell, papillary (Type I and II) and chromophobe the most frequent ones [36, 37]. The clear cell Renal Cell Carcinoma (ccRCC) not only is the most common but also the most aggressive subtype, presenting intense vascularity and high expression of angiogenic factors [38, 39]. Although this tumor is mainly associated with sporadic cases, it was the uncommon hereditary form, linked to *von Hippel-Lindau* (*VHL*) gene mutation, that gave insight in the mechanism involved in this tumor development [40]. Indeed, the majority of sporadic and hereditary (VHL syndrome) ccRCC cases lacks functional VHL due to loss, mutation or hipermethylation of *VHL* gene [41].

The tumor suppressor gene *VHL* is located on the short arm of chromosome 3 (3p25-p26) and deletions in this region, which often involves the whole short arm of chromosome 3, are observed in approximately 90% of all ccRCC [42]. *VHL* encodes a protein (pVHL) that is the substrate-specifying component of the multiprotein E3 ubiquitin ligase ECV (Elongins B and C/Cullin 2/pVHL). pVHL catalyzes the poly-ubiquitylation of prolyl-hydroxylated substrates for subsequent degradation via 26S proteasome [41]. One of the most important protein targets of

pVHL is the Hypoxia Inducible Factor (HIF) family of transcription factors: HIF1α, HIF2α and HIF3α [43]. Once expressed in the cell, HIFα dimerizes with the constitutive expressed subunit HIFβ to form an active heterodimeric HIF transcription factor, and this complex translocates to the nucleus. Under normoxic conditions, HIFα subunits are hydroxylated and recognized by pVHL that mediates its proteasome degradation (Figure 3 A) [43, 44]. On the other hand, since one of the early molecular events of ccRCC is the loss of pVHL, HIF accumulates and binds to hypoxia-responsive elements (HREs) located in the promoter/enhancer regions of numerous hypoxia-inducible genes in order to initiate various adaptive responses to hypoxia in non-hypoxic conditions. These genes are involved in pathways responsible for angiogenesis (VEGF), proliferation (transforming growth factor α –TGFα, platelet-derived growth factor β –PDGFβ), glucose metabolism (glucose transporter 1 – GLUT1, phosphoglycerate kinase – PGK, and lactose dehydrogenase A – LDHA), erythropoiesis (erythropoietin – EPO) and metastasis (chemokine (C-X-C motif) receptor 4 – CXCR4) (Figure 3 B) [39, 45, 46]. To date, over 70 genes have been validated as direct HIF targets, indicating the complex network triggered upon HIF activation [47]. One of the activated genes is TGFα, that like Epidermal Growth Factor (EGF), is involved in cellular proliferation induction by activation of the Epidermal Growth Factor Receptor (EGFR/ErbB/Her1) (Figure 3 C) [48]. The ErbB/HER family contains four tyrosine kinase receptors (TKR), the EGFR/ErbB1/Her1, ErbB2/Her2/Neu, ErbB3/Her3, and ErbB4/Her4 consisting of an extracellular ligand-binding domain, a single transmembrane domain, an uninterrupted tyrosine kinase domain, and a cytoplasmic tail [49]. These TKRs are able to form homo- or heterodimers upon ligand binding [50, 51]. Downstream ErbB signaling pathways include PI3K/AKT, MAPK/ERK, PLCγ, and signal transducers and activators of transcription (STAT). All of these pathways are interconnected and overlapping [52, 53]. Overall, they regulate apoptosis, cell cycle progression, cytoskeletal rearrangement, differentiation, development, immune responses and transcription regulation [54].

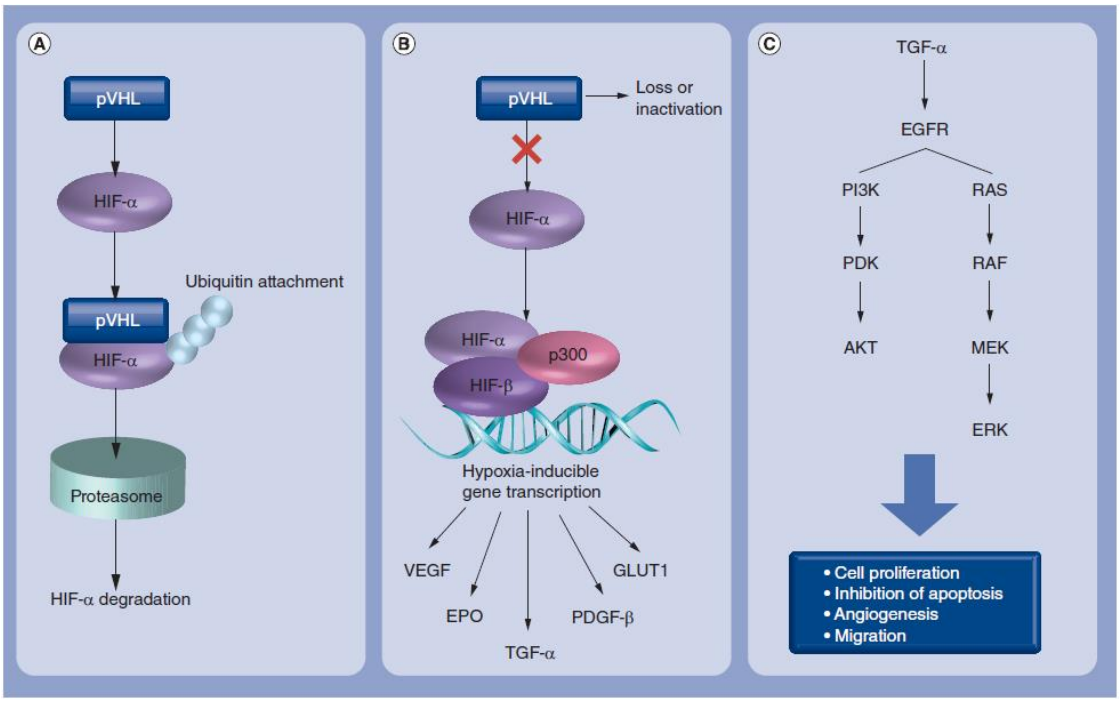

Figure 3 – Signaling pathways involved in ccRCC pathophysiology. A) *VHL* pathway under normoxic conditions: pVHL targets HIFα for proteasome degradation. B) *VHL* pathway under hypoxic conditions: *VHL* loss leads to HIFα accumulation in the nucleus and consequent binding of transcription factors, which trigger hypoxic responses. C) EGFR pathways activation by TGFα and its physiologic consequences. Adapted from Dias *et al.* 2013 [58].

EGFR is a well-known TKR often dysregulated in several malignancies. EGFR overexpression has been described in head and neck, breast, bladder, prostate, kidney, non-small cell lung cancer and glioma tumors [50]. In RCC, EGFR overexpression has been associated with higher tumor grade and poor overall survival [55]. The pVHL is also responsible for activated EGFR turnover and further degradation in the proteasome, thus during ccRCC development this process could be compromised leading to an increase of EGFR protein levels [56]. Additionally, Zhou and colleagues observed that EGFR half-life in cells with functional pVHL was approximately 1 hour, whereas in cells *VHL*-defective EGFR half-life triplicated [57]. Also, it has been described that hypoxia upregulates EGFR and prolong its activation through retention in the endocytic trafficking [56].

VHL loss and consequent increase in EGFR half-life and overproduction of TGFα, leads to the activation of different pathways in an autocrine and/or paracrine fashion. Collectively, these findings demonstrate the importance of EGFR-related pathways in ccRCC progression. Furthermore, *VHL* impairment and EGFR pathways dysregulation in ccRCC have been associated with altered levels of microRNAs (miRNAs), a class of small non-coding RNAs of 19 to 25 nucleotides [58, 59]. Mature miRNAs result from a sequential processing that initiates at the

nucleus with the transcription of a primary RNA (pri-miRNA) by RNA polymerase II [60]. The pri-miRNA is processed by two RNase III endonucleases, Drosha and Dicer, members of the Argonaute family. First, Drosha creates a pre-miRNA that is then exported to the cytoplasm and cleaved by Dicer into a mature 22 base pair double-stranded miRNA [61]. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), becoming functional [62]. MiRNAs are then able to bind to complementary sequences in the 3' untranslated regions (3' UTR) of their target mRNAs and induce mRNA degradation or translation repression, thus regulating several biological processes including cell mobility, differentiation, proliferation and apoptosis [63, 64]. Moreover, it has been shown that miRNAs are aberrantly expressed in cancer, and they have been considered a novel class of oncogenes or tumor suppressor genes [65]. OncomiRNAs are known to downregulate tumor suppressor genes, and have been reported to be overexpressed in multiple miRNA-profiling studies performed in different tumor models [65]. Whilst tumor suppressor miRNAs are responsible for downregulating oncogenes, and are mostly underexpressed in cancer [65, 66]. Some studies, mainly array-based ones, have demonstrated that a considerable number of miRNAs are dysregulated in ccRCC, and a few have been associated with altered signaling pathways in this malignancy [59, 67-69].

In ccRCC, the crucial VHL/HIF/EGFR axis can be regulated by p53 action, since MDM2 (an E3 ubiquitin–protein ligase) mediates HIFα degradation [70]. Additionally, it has been hypothesized that p53 levels could be regulated by miRNA-1233, becoming a potential central key player in the regulation of these signaling pathways (Figure 4) [58]. Recent studies developed by Dias and colleagues showed that various miRNAs were dysregulated in ccRCC, one of which miRNA-1233 [71]. MiRNA-1233 levels were upregulated in plasma samples of ccRCC patients when compared to healthy subjects. Moreover, higher levels of this miRNA correlated with tumor grade and metastatic disease, suggesting the importance of miRNA-1233 in ccRCC dissemination [71]. Information concerning miRNA-1233 function is scarce. MiRNA-1233 is located on chromosome 15q14 and was classified as an oncomiRNA for the first time by Wulfken and colleagues. They have confirmed upregulated levels of miRNA-1233 in serum samples of RCC patients compared to healthy controls [72]. Collectively, these findings demonstrate that miRNA-1233 could be a promising candidate to be targeted in ccRRCC. Furthermore, using *in silico* analysis Wulfken and colleagues found several potential targets for miRNA-1233, some of which are tumor suppressor genes, including *TP53* [72]. The p53 transcription factor (encoded by *TP53*) is a key regulator of various signaling pathways tumor suppressor-related including: cell-cycle arrest, DNA repair, senescence, hypoxia-induced apoptosis, inhibition of angiogenesis and vascularization, among others [73]. In fact, p53 is known to limit tumor vascularization in three different ways: 1)

increasing anti-angiogenic factors production such as thrombospondin-1; 2) directly inhibiting HIF by targeting the HIF1α subunit for MDM2-mediated ubiquitination and proteasome degradation; and 3) negatively regulating the expression of genes encoding pro-angiogenic factors like VEGF [70, 74, 75]. These findings indicate that p53 regulates HIF-related pathways. In tumor cells p53 is often downregulated, amplifying the normal HIF-dependent responses to hypoxia. This is achieved through production of VEGF and TGFα, contributing to the angiogenic switch activation and to the EGFR-related pathways activation, respectively. Taken together, in a ccRCC context, high levels of miRNA-1233 could lead to p53 downregulation, ultimately promoting a continuous state of hypoxia, cell survival, cell proliferation and angiogenesis.

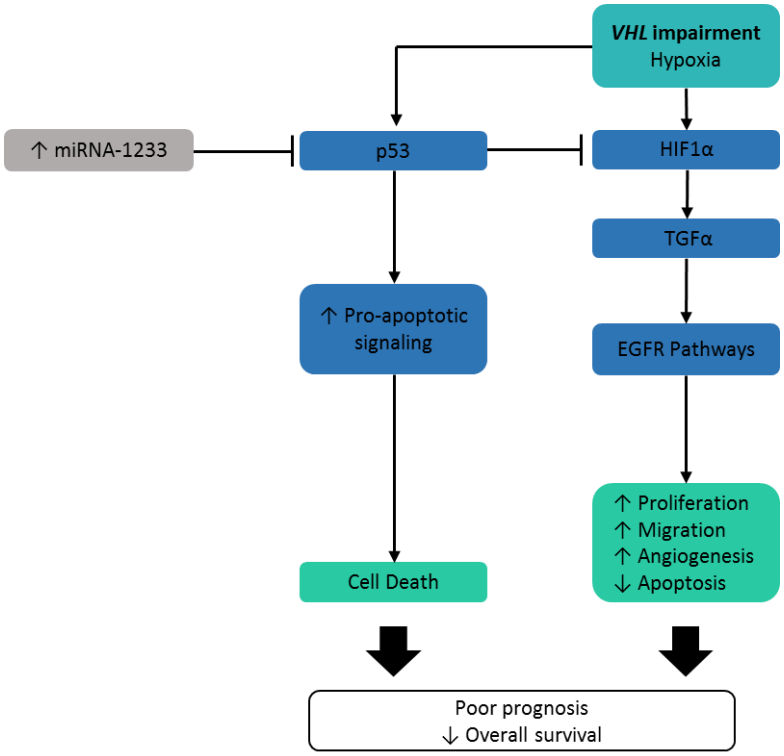

Figure 4 – Proposed model of miRNA-1233 role during ccRCC development according to the results obtained by Dias *et al.* [71].

In recent years, the increasing knowledge of the pathways involved in ccRCC initiation and progression has allowed the development of promising targeted therapies. The implication of certain genes such as *VHL*, *HIF1α*, *EGFR* and *VEGF* in ccRCC carcinogenesis led to the development of targeted therapies that can fall in two categories: TKI and monoclonal antibodies. TKI are small molecules that can target a wide range of TKR such as sorafenib (Nexavar®) and sunitinib (Sutent®), they can be angiogenesis inhibitors for example axitinib (Inlyta®) and pazopanib (Votrient®), and also inhibitors of mammalian target of rapamycin (mTOR), for instance temsirolimus (Torisel®) and everolimus (Afinitor®). Additionally, antibodies

like bevacizumab (Avastin®) can be used to inhibit angiogenesis by targeting VEGF [21]. However, a subset of patients (approximately 25%) do not experience any clinical benefit from these targeted therapies [33]. Moreover, in the majority of cases, patients initially respond to therapy but end up developing resistance after 5 to 11 months upon treatment, leading to cancer progression [34]. These observations highlight the importance of developing new therapeutic approaches in order to overcome therapy limitations currently faced in the clinic. Over recent years, many groups have focused their research in the discovery and development of new therapeutic strategies. As a result, a new promising specific delivery system has been identified: the exosomes [76].

1.4 Exosomes

The best characterized EVs are the exosomes, ranging in size from 30 to 150 nm in diameter [77]. Exosomes are derived from the endolysosomal pathway and are formed within multivesicular bodies (MVBs), being released by cells upon fusion of MVBs with the plasma membrane [77]. Most cell types produce exosomes, including tumor cells, and release them into the interstitial space, which ultimately enter circulation [78]. Therefore, exosomes can be found in different body fluids such as blood, saliva, urine, breast milk, amniotic fluid, ascites, cerebrospinal fluid, bile, and semen [77, 79]. This type of extracellular vesicles is enriched in cholesterol, sphingomyelin and ceramide as well as lipid-raft associated proteins [78, 80]. Proteomic studies showed that nearly all exosomes, regardless of the cell type from which they originate, contain a similar membrane composition. However, the exosomal lumen is determined by the cell type of origin and includes proteins, DNA and RNAs (mRNAs, miRNAs and other non-coding RNAs) [81, 82].

Numerous studies have tried to identify the role of exosomes both in normal and pathological conditions [83]. Regarding the exosomes role in the immune system, it is known that they can trigger an immune response through T-cell activation either by a direct antigen presentation or by an indirect presentation through transfer of antigenic peptides to antigen-presenting cells [84, 85]. In an infectious disease context, pathogen-released exosomes are known to carry specific virulence factors that contribute to spread the infection [86, 87]. As a consequence, exosomes can either expand or contain the infection, hence being beneficial for the pathogen or the host. Exosomes were also proven to be involved in the normal development of the

nervous system and in its physiology, including synaptic plasticity [88]. Though, they were also associated with the generation and progression of many neurodegenerative diseases since they are able to shuttle toxic agents like misfolded proteins from unhealthy neurons to neighboring cells [89]. For instance, amyloid-β-derived peptides and α-synuclein, respectively linked to Alzheimer's and Parkinson's diseases [90, 91].

However, it is in the field of cancer that exosomes have been extensively studied. Since the exosomal lumen reflects the content of the cell from which they originate, several studies have focused on their potential use as a diagnostic or prognostic tool. Exosomes provide means for a liquid biopsy that is minimally invasive. Indeed, as demonstrated by Melo and colleagues, exosomes derived from pancreatic cancer patients serum were positive for glypican-1 (GPC1) and able to distinguish healthy subjects and patients with a benign pancreatic disease from patients with early- and late-stage pancreatic cancer [92]. Furthermore, due to exosomes intrinsic ability of horizontal cargo transfer and high stability in circulation, they can interact with neighboring or distant cells and phenotypically reprogram recipient cells [93]. There are three main possible mechanisms of intracellular communication by exosomes (Figure 5) [35]. First, exosomal membrane proteins can interact with receptors in a target cell and activate different signaling pathways. Second, proteases in the extracellular space can cleave exosomal membrane proteins, leading to a cleaved fragment-target cell surface receptor interaction. This mechanism also activates intracellular signaling. Finally, exosomes can fuse with the target cell membrane

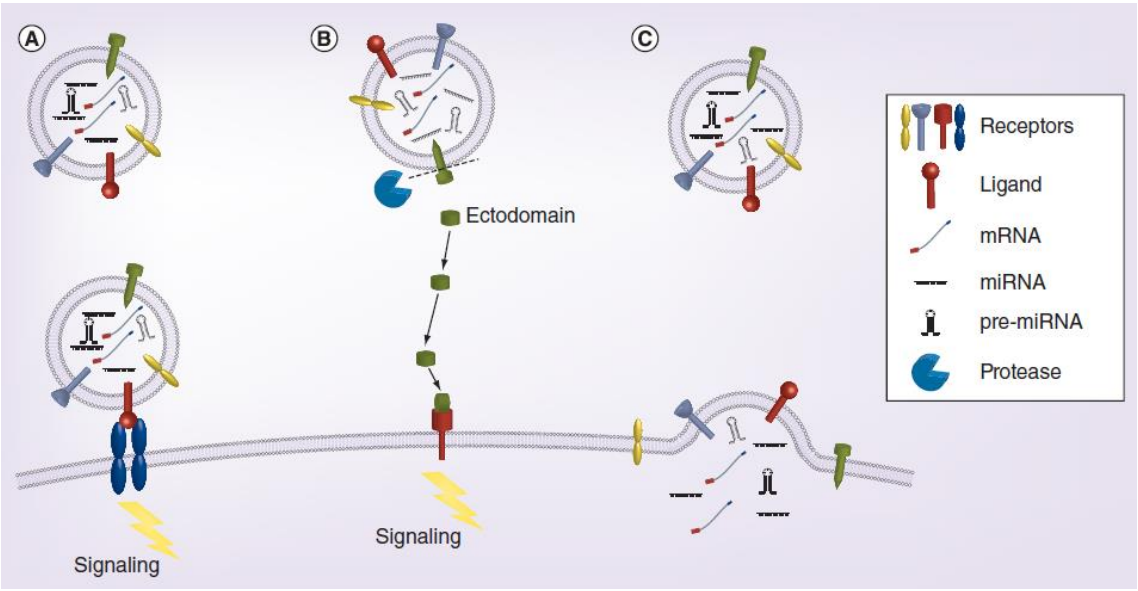

Figure 5 – Possible mechanisms of exosome-target cell interaction. A) Interaction between exosomal membrane proteins and target cell receptors leads to intracellular signaling activation. B) Interaction between an ectodomain, formed after protease cleavage of exosomal membrane proteins, and target cell receptors also leads to signaling pathways activation. C) Exosomes fusion with the recipient cell membrane and content release to the intracellular space. Adapted from Adem *et al.* [35].

and release their content, such as mRNAs and miRNAs, which can alter gene expression and protein translation of the recipient cell [78].

Recently, various studies have showed the role of exosomes in tumor initiation, through driving the formation of a pre-metastatic tumor niche [94]. Also, increasing evidence demonstrates that tumor-secreted exosomes are able to stimulate tumor progression through different manners. It has been shown that exosomes can promote immune escape by modeling T cell activity and are able to regulate the hematopoyetic system, including lineage-specific differentiation of bone marrow precursors and dendritic cell (DC) function [95-98]. Their ability to induce cell proliferation and angiogenesis, contributing to tumor progression has also been established [99, 100]. Additionally, tumor-derived exosomes containing specific factors can stimulate matrix metalloproteinase expression in fibroblasts and consequently promote matrix remodeling and ultimately facilitate tumor invasion and metastasis [101].

Despite latest findings and intensive efforts, the exosomes role in both normal and pathological conditions remains poorly understood. Further investigation is needed in order to clarify the precise way these extracellular vesicles can mediate cell-to-cell communication and affect the initiation and development of such a complex and heterogeneous disease like cancer.

1.5 Exosomes-based therapy

Exosomes have been explored for usage in therapy of a wide range of diseases. Numerous studies have demonstrated the possibility of using exosomes as vaccines for allergic or infectious diseases, and its applicability in autoimmune diseases treatment [102, 103]. Additionally, these extracellular vesicles are being extensively studied in cancer-related therapeutics. More specifically, exosomes are being used to either increase innate and adaptive immune responses against the tumor or to deliver therapeutic agents in a cancer-specific way [102].

Regarding the use of exosomes in immunotherapy, patient-specific exosomes secreted by DC (dexosomes) can be loaded with tumor antigen-derived peptides and used as a vaccine to generate antitumor immune responses [104]. Phase I clinical trials using dexosomes in the treatment of metastatic melanoma and advanced non-small cell lung cancer showed that dexosome immunotherapy was feasible, safe, efficient in induction of both innate and adaptive immune responses, promoted disease stabilization increasing long-term survival in several

patients [105, 106]. A phase II clinical trial is currently on going for the treatment of non-small cell lung cancer with a dexosome-based vaccine [107]. Moreover, ascites-derived exosomes from colorectal cancer patients were also shown to be safe, nontoxic, and tolerable when used as a cancer vaccine. Furthermore, in advanced colorectal cancer patients when combining ascites-derived exosomes with granulocyte-macrophage colony-stimulating factor (GM-CSF) it was possible to efficiently induce potent antigen-specific antitumor immunity [108]. Even though major advances have been made in recent years, it should be noted that the application of exosomes as a cancer vaccine requires further research in order to completely clarify the mechanisms underlying this sort of approach, and more importantly, to standardize procedure methods.

More recently, exosomes have been seen as an alternative *in vivo* delivery system to other therapeutic nanoparticles such as liposomes and polymers [109-111]. The intrinsic ability of exosomes to interact and modulate recipient cells through the shuttle of small molecules and also their high stability in circulation makes them an extremely promising candidate to an *in vivo* delivery of therapeutic agents [35, 112]. In fact, a phase I clinical trial is investigating the ability of plant-derived exosomes to deliver curcumin to normal and colon cancer tissue, since previous studies demonstrated that curcumin has a strong inhibitory effect on the growth of colon cancer cell lines by inhibiting mTOR signaling pathway [113, 114].

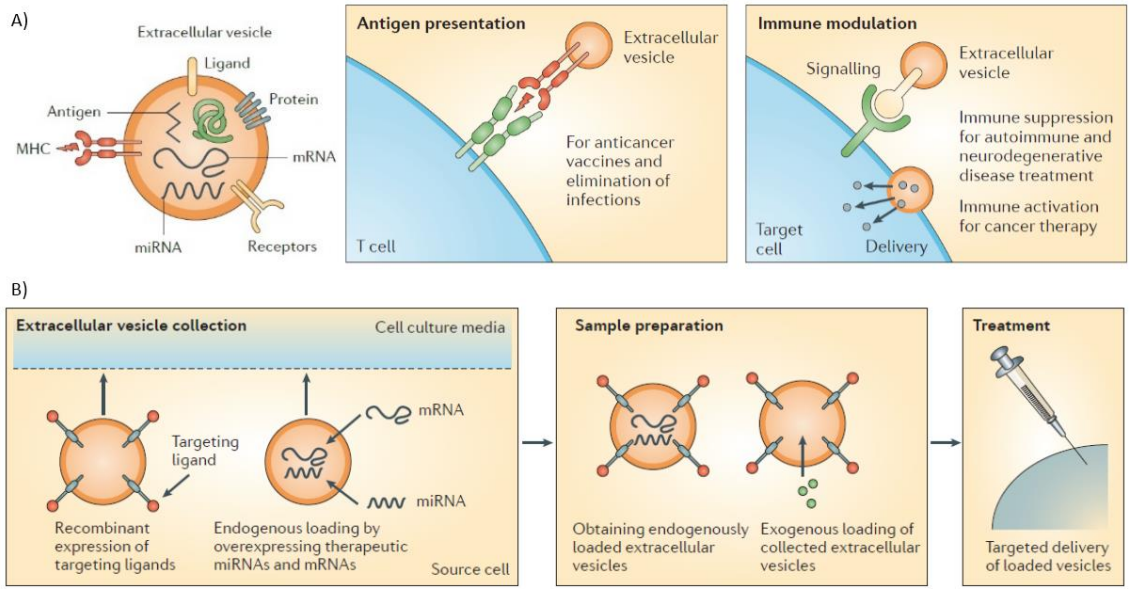

Figure 6 – Emerging exosome-based strategies for cancer therapy. A) Immunomodulatory effects of exosomes can include antigen transfer and presentation to T cells (anticancer vaccines). B) Exosomes can be engineered to express certain cell type-specific ligands present on their surface by expressing plasmid fusion constructs comprising targeting ligands fused to extracellular vesicle transmembrane proteins. Drug loading can be achieved either endogenously (for example by overexpressing a miRNA in source cells) or exogenously (co-incubation or through electroporation). Finally, after collection and purification, exosomes can be tested in *in vitro* or *in vivo*. Adapted from Andaloussi *et al.* [115].

In order to take full advantage of RNA drugs, an efficient, tissue-specific and non-immunogenic delivery technology must be developed. Exosomes are the most qualified candidates so far due to their biocompatibility, the fact they are immunologically inert if derived from appropriate cells, and able to protect sensitive drug molecules such as RNA, enhancing drug solubilization and surpassing the blood-brain barrier [76, 115]. In addition, they are natural RNA carriers making them a valuable tool for RNA interference (RNAi) delivery. RNAi is a phenomenon in cell biology whereby small RNA molecules (small interfering RNA (siRNA) and miRNA) control gene expression post-transcriptionally [115, 116].

Alvarez-Erviti and colleagues were the first to provide evidence of the RNA-transporting capacity of exosomes [117]. In their study, DC-derived exosomes expressing Lamp2b, an exosomal membrane protein that reduces immunogenicity, fused to the neuron-specific RVG peptide were loaded with BACE (a therapeutic target in Alzheimer's disease, since it is involved in β-amyloid plaque formation) siRNA. These engineered vesicles were injected in mice and the siRNA was delivered specifically to the brain. Consequently, decreased *BACE* gene expression was observed in neurons, microglia and oligodendrocytes [117]. Regarding the use of exosomes containing exogenous miRNAs, these molecules can fall in two main categories: miRNA mimics or antagomiRNAs [118]. In order to restore the tumor suppressor levels of a certain miRNA, synthetic miRNA-like molecules called miRNA mimics can be used. MiRNA mimics are small, chemically modified (2'-O-methoxy) RNA duplexes that are processed into single-strand form inside the cells and loaded into RISC to achieve the downstream inhibition of the target mRNAs [118, 119]. OncomiRNAs, often overexpressed in tumor cells, can be inhibited using various synthetic modified antisense oligonucleotides such as anti-miRNA oligonucleotides (AMOs), locked nucleic acid (LNA) anti-miRNAs, miRNA sponges, miRNA masks, and small-molecule inhibitors of miRNAs [118, 120]. Since miRNAs regulate several cellular processes and are often dysregulated in cancer cells, agents that modulate miRNA activity could potentially produce cancer-specific effects [65]. The combination of engineered exosomes with RNAi-based therapy could become a new therapeutic approach for cancer treatment [76]. Indeed, Ohno and colleagues demonstrated the ability of GE11 positive ($GE11^+$) exosomes to deliver a miRNA let-7a mimic to breast cancer cells *in vivo* [121]. They engineered exosomes in order to express a fusion protein on their membranes, using the PDGF Receptor (PDGFR) transmembrane domain to anchor the protein to the exosomal membrane and expose the GE11 peptide to the extracellular space. The GE11 is a synthetic peptide that binds specifically to EGFR and is markedly less mitogenic than EGF [122]. In this study, breast cancer cells internalized the engineered exosomes through an EGFR-dependent mechanism. Additionally, miRNA-let-7a, a

tumor suppressor miRNA frequently downregulated in breast cancer, was introduced in $GE11^+$ exosomes and used to treat an orthotropic breast cancer model in $RAG2^{-/-}$ mice [123]. This treatment resulted in an efficient delivery of $GE11^+$ exosomes cargo to breast cancer cells with consequent tumor growth suppression and no major organ damage was detected in the injected mice [121]. Overall, Ohno and colleagues showed that $GE11^+$ exosomes could be used as a new drug delivery system in an EGFR-overexpressing cancer model.

It is known that EGFR is frequently overexpressed in a wide range of human tumors of epithelial origin including renal malignancies such as ccRCC [124]. Also, ccRCC patients end up developing resistance to chemotherapy, radiotherapy and to the targeted therapies used in ccRCC treatment [34, 35]. Hence, the development of new therapeutic approaches, as promising as this exosome-based one, are crucial to overcome limitations currently faced in this malignancy treatment. Collectively, these studies emphasize the potential use of engineered exosomes for the delivery of RNAi drugs like miRNA mimics or antagonists and its application in nanomedicine. Therefore, elucidating the mechanisms subjacent to ccRCC initiation and progression like the EGFR dysregulation and the potential involvement of miRNA-1233 in p53/HIF/EGFR pathways will contribute to the development of a new strategy for ccRCC treatment based on $GE11^+$ exosomes, ultimately loaded with a miRNA-1233 antagonist.

2. Objectives

2.1 Main Objective

Study the potential applicability of $GE11^+$ exosomes as a promising RNAi therapy delivery vehicle in ccRCC.

2.2 Specific Objectives

- *In vitro* characterization of EGFR and ERK pathway activation in a normal proximal tubule epithelial renal cell line and in a metastatic ccRCC cell line.
- *In vitro* characterization of miRNA-1233 and *TP53* mRNA expression levels in a normal proximal tubule epithelial renal cell line and in a metastatic ccRCC cell line.
- Analysis of the functional association between miRNA-1233 and *TP53* mRNA in a metastatic ccRCC cell line.
- Production of engineered exosomes that display a synthetic EGFR ligand, the GE11 peptide, on the membrane.
- *In vitro* characterization of the uptake levels of $GE11^+$ exosomes by a normal proximal tubule epithelial renal cell line and a metastatic ccRCC cell line.

3. Material and Methods

3.1 Cell lines

For this study, four cell lines were used: HEK293T, HKC-8, HK-2 and FG-2. HEK293T cell line is derived from human embryonic kidney cells and has an epithelial morphology. Both HKC-8 and HK-2 are immortalized proximal tubule epithelial cell lines derived from normal adult human kidney. FG-2 is classified as a human kidney metastatic clear cell carcinoma cell line.

To start cell culture a cryopreserved vial of each cell line used in this study was thawed. The HEK293T and FG-2 cell lines were maintained in RPMI 1640 (1X) medium (Gibco®), supplemented with 10% of FBS (Fetal Bovine Serum) (Gibco®), and 1% penicillin-streptomycin (Gibco®). The HKC-8 and HK-2 cell lines were kept in DMEM/F12 medium (Gibco®), supplemented with 10% of FBS (Gibco®), ITS (Insulin-transferrin-selenium) (Sigma-Aldrich), EGF (Sigma-Aldrich), hydrocortisone (Sigma-Aldrich), and 1% penicillin-streptomycin (Gibco®). All cell lines were maintained in a 5% CO_2 incubator at 37°C.

3.2 Plasmids propagation

The plasmids used in the present study were: pDisplay and pDisplay-GE11, kindly provided by Doctor Shin-ichiro Ohno from Tokyo Medical University, Japan, and mCherry kindly given by Doctor Carlos Reguenga from Porto's Medical School. The propagation of the plasmids was achieved by transforming the following competent bacteria NZYstar competent cells (nzytech). To extract and purify the plasmids of interest the kit NucleoBond® Xtra Midi (Machery-Nagel) was used according to the manufacturer's protocol. The DNA concentration and purity related to each plasmid were measured at 260 and 280 nm using the NanoDrop® ND-1000 spectrophotometer.

3.3 Transfections

Regarding HEK293T cell line transfection the plasmids used were: pDisplay, pDisplay-GE11, and mCherry. HEK293T cell line was transiently transfected separately with each of the three DNA plasmids mentioned above (2µg DNA/5 × 10^5 cells) in adherent cells, using Invitrogen Lipofectamine® 2000 (Invitrogen) according to the manufacturer's instructions.

The FG-2 cell line was seeded in a 24-well plate with a confluence of 1.5 × 10^5 cells per well. Cells were transfected with a scrambled sequence (kindly given by Doctor Germana Zaccagnini from IRCCS-Policlinico San Donato, Italy) or with *miR*Vana® miRNA-1233 mimic (has-miR-1233-3p, MC13610, Applied Biosystems®), both at a final concentration of 60nM, using ScreenFect®A (InCella) transfection reagent, according to the manufacturer's instructions. 24 or 48h after transfection, cells were trypsinized and both mRNA and miRNA were isolated and purified as previously described for further analysis by real-time PCR of miRNA-1233, RNU-6B, TP53 and B2M levels. This experience was performed three times.

3.4 Exosomes production and isolation

HEK293T cells were grown for 72h in FBS-depleted exosomes RPMI media. Exosomes were isolated from cells supernatant. The medium was initially centrifuged at 12 000 rpm for 10 min and then filtered through a 0.22 µm filter (GE Healthcare Whatman™). Next, the exosomes were pelleted via *overnight* ultracentrifugation at 100 000 *x g* and 4°C using the Optima™ L-80 XP ultracentrifuge, Beckman Coulter, and the rotor SW 41.

3.5 Western-blot

HKC-8, HK-2 and FG-2 cell lines were plated in a 6-well plate (5 × 10^5 cells per well). Cells were washed with PBS (1X) (pH 7.4) (Fisher BioReagents™) and lysed with RIPA buffer (amresco) supplemented with protease inhibitor cOmplete (Roche®) and phosphatase inhibitor phenylmethanesulfonyl fluoride (Sigma-Aldrich). The cell lysates were incubated in ice for 30

min following a 30 min centrifugation at 17 000 *x g* and 4°C. Subsequently, total protein concentrations in the supernatant were determined using an adaptation to the Lowry's method (DC™ Protein Assay Reagent, BIO-RAD®) according to the manufacturer's instructions. For western-blot analyses 15µg proteins were separated by 10% (w/v) sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis. The molecular weight estimation of the obtained bands was made using Precision Plus Protein™ Dual Color Standards (BIO-RAD®). The separated proteins were transferred onto nitrocellulose membranes 0.2µm (GE Healthcare®) using a wet electrophoretic transfer for 2h at 100V. The membrane was blocked for 1h at room temperature with 5% non-fat dry milk in PBS/0.1% Tween 20. The following primary antibodies were added and incubated *overnight* on a shaker at 4°C: anti-EGFR (dilution 1:1000, Cell Signalling®), anti-pEGFR (dilution 1:500, Cell Signalling®), anti-ERK1/2 (dilution 1:1000, Cell Signalling®), anti-pERK1/2 (dilution 1:1000, Cell Signalling®), and anti-β-actin (dilution 1:5000, Sigma-Aldrich). Afterwards, horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:10 000, Cell Signalling®) were incubated for 1h at room temperature.

Regarding HEK293T-related western-blots, protein extraction from HEK293T cells followed the procedure above mentioned while for protein isolation from HEK293T exosomes, the exosomes pellet were lysed in 8 M urea and 2.5% SDS, incubated in ice for 30 min following a 30 min centrifugation at 17 000 *x g* and 4°C. After protein isolation and quantification as previously described, 15µg proteins from HEK293T cells and 20µg proteins from HEK293T exosomes were separated by 15% (w/v) SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred onto Polyvinylidene Difluoride (PVDF) membranes 0.2µm (BIO-RAD®) using a wet electrophoretic transfer for 1h at 65V and 4°C. The membranes were blocked for 1h at room temperature with 5% non-fat dry milk in PBS/0.1% Tween 20. Next, the following primary antibodies were added and incubated *overnight* on a shaker at 4°C: anti-c-Myc (dilution 1:200, 9E10, Santa Cruz Biotechnology®), anti-hemagglutinin (anti-HA) (dilution 1:1000, Sigma-Aldrich) and anti-β-actin (dilution 1:5000, Sigma-Aldrich). Then, blots were incubated for 1h at room temperature with the respective HRP-conjugated secondary antibodies (dilution 1:10 000, Cell Signalling®).

All washes after antibody incubations were done on an orbital shaker, four times at 10 min intervals, with PBS/0.1% Tween 20. To finalize, all membranes were incubated with Clarity™ Western ECL Substrate (BIO-RAD®), according to the manufacturer's recommendations, for visualization on a ChemiDoc machine using ImageLab (BIO-RAD®) or development using GE Healthcare Amersham™ Hyperfilm™ ECL.

3.6 Immunofluorescence

HEK293T cells were plated 24h prior to the transient transfection with pDisplay-GE11 plasmid in a 24-well plate (1.1×10^5 cells per well). Each well had been previously coated with a coverslip and treated with a solution of 10% Poly-L-Lysine (Sigma-Aldrich) for 1h at room temperature. The cell medium was replaced 8h after transfection. The immunofluorescence protocol was started 36h after transfection and cells were washed with cold PBS (1X) (Fisher BioReagents™) and fixed in 4% paraformaldehyde (PFA) (Sigma Aldrich) for 15 min at room temperature. Afterwards, a quenching solution of glycine 1M was added to cells. The blocking was performed by incubating cells for 45 min with a solution of 10% FBS (Gibco®). Cells were incubated *overnight* with the primary antibody anti-c-Myc (dilution 1:200, 9E10, Santa Cruz Biotechnology®) or anti-HA (dilution 1:500, Sigma-Aldrich) at 4°C. Next, cells were incubated for 45 min with the secondary antibodies anti-IgG mouse or anti-IgG rabbit Alexa-Fluor® 488 (dilution 1:1500, Invitrogen®). All the washing steps were made using PBS (1X) (Fisher BioReagents™) and by incubating cells for 5 min at room temperature more than once. Then, coverslips were mounted using a drop of fluoroshield mounting medium with DAPI (abcam®). The coverslips were sealed with nail polish to prevent drying and movement under microscope and samples were kept at 4°C protected from light until observation using laser scanning confocal microscope Leica TCS SP5 II (Leica Microsystems, Germany).

3.7 Transmission electron microscopy immunogold

The engineered exosomes produced by HEK293T cells were labeled and then visualized using transmission electron microscopy (TEM) according to Théry *et al.* [125]. Briefly, the pellet of exosomes obtained after ultracentrifugation was resuspended in 100µL of 2% PFA. Next, 5µL of this mixture was transferred onto Formvar-carbon coated electron microscopy grids (Electron Microscopy Sciences). The membranes were incubated for 20 min. Grids were transferred to a 50µL drop of saline solution with the sample membrane side facing down and were washed twice for 2 min. Afterwards, grids were moved to a 50µL drop of saline solution/Glycine 50mM for 3 min and were washed four times. Grids were blocked for 15 min in saline solution/BSA 5%. Then, samples were incubated for 45 min with the primary antibody at a final dilution of 1:20. For this step, grids were transferred to a 5µL drop of saline solution/BSA 1% with the primary

antibody anti-c-Myc (dilution 1:20, 9E10, Santa Cruz Biotechnology®) or CD81 (dilution 1:20, abcam®). Next, grids were washed in a 50µL drop of saline solution/BSA 0.1% for 3 min for a total of six washes. The secondary antibody (anti-mouse gold or anti-rabbit gold) (dilution 1:20, abcam®) was diluted in saline solution/BSA 0.5% at a final volume of 5µL and incubated for 30 min at room temperature. Grids were washed in a 50µL drop of saline solution/BSA 0.5% for 3 min for a total of six washes. Grids were transferred to a 50µL drop of 1% glutaraldehyde for 5 min before transferring to a 50µL drop of distilled water for 2 min. This was repeated 7 times for a total of 8 water washes. To contrast samples, grids were transferred to a drop of uranyl acetate for 5 seconds. Grids were left to dry and observed with JEM 1.011 transmission electron microscope at 80 kV.

3.8 Exosomes staining

Exosomes were labeled with the green fluorescent dye PKH67 (Sigma-Aldrich) according to the manufacturer's recommendations. Briefly, the pelleted exosomes obtained from HEK293T culture media were resuspended in 1ml Diluent C, and 4µl PKH67 was diluted in another 1ml Diluent C. These two solutions were mixed gently for 5 min, after which 2ml 2% BSA was added to bind the excess dye. The mixture was subsequently ultracentrifuged *overnight* at 100 000 *x g* and 4°C and the final pellets were resuspended in complete medium.

3.9 Flow cytometry

In order to evaluate the uptake levels of the engineered fluorescent labeled exosomes by normal and tumor renal cell lines, flow cytometry was performed. FG-2 and HK-2 cells were plated in a 12-well plate (2 x 10^5 cells per well). After 24h, the pellet of exosomes correspondent to one well of a 6-well plate was used to treat cells plated in three wells of a 12-well plate. Both cell lines were treated with the engineered fluorescent labeled exosomes for 4 hours at 37°C or 4°C. The experiment was performed in triplicates.

3.10 RNA extraction and cDNA synthesis

Regarding HKC-8 and FG-2 cell lines, when the desired confluence was achieved (80-90%) cells were trypsinized, using 0.05% (w/v) trypsin-EDTA (1×) (Gibco®) and counted using a Neubauer chamber and Tripan-Blue dye (Gibco®). After counting, approximately two million cells were centrifuged for 3 min at 1200 rpm to form a pellet for mRNA or miRNA extraction and the remaining cells were kept in culture under the conditions above cited. This procedure was repeated ten times for each cell line.

Total RNA extraction and purification was performed using the GeneJET™ RNA purification kit (Thermo Scientific®), according to the manufacturer's instructions. After isolation, NanoDrop® ND-1000 spectrophotometer was used to determine RNA concentration and purity by measuring absorbance at 260 and 280 nm. The RNA samples were then used as templates for cDNA synthesis using the High-Capacity RNA-to-cDNA kit (Applied Biosystems®) in accordance with the manufacturer's protocol.

Regarding miRNA extraction and purification, GRS microRNA kit (Grisp®) was used according to the manufacturer's instructions. After isolation, miRNA concentration and purity were measured at 260 and 280 nm using the NanoDrop® ND-1000 spectrophotometer. Then, the RNA samples served as templates for cDNA synthesis using the TaqMan®MicroRNA Reverse Transcription Kit (Applied Biosystems®) and sequence-specific stem-loop primers for miRNA-1233, RNU-48 and RNU-6B.

3.11 Real-time PCR relative quantification

The mRNA and miRNA expressions were analyzed by quantitative real-time PCR, using a StepOne™qPCR Real-Time PCR machine. Concerning mRNA analysis, each reaction contained 1X Master mix (Applied Biosystems®), 1X specific probes for each transcript in study (TaqMan® Gene Expression Assays: EGFR: Hs01076078; TP53: Hs01034249, B2M (Beta-2-Microglobulin) Endogenous control: 4333766, Applied Biosystems®), and cDNA sample. B2M was used to normalize the results since it presents a constant expression level in the cell lines in study.

Regarding miRNA analysis, each reaction contained 1X Master mix (Applied Biosystems®), 1X probes (TaqMan® microRNA Expression Assays: miRNA-1233: TM-002768, RNU-48: TM-001006,

RNU-6B: TM-001093, Applied Biosystems®), and cDNA sample. RNU-48 was used as endogenous control when comparing HKC-8 and FG-2 samples, whereas RNU-6B was used as endogenous control when comparing samples from FG-2 cells only (miRNA-1233 mimic experiments). These endogenous controls were chosen upon previous protocol optimization, in order to normalize the results since they are expressed in a constant manner.

Data analysis was made using StepOneTM Sofware v2.2 (Applied Biosystems®) with the same baseline and threshold set for each plate in order to generate threshold cycle (Ct) values for all the mRNA or miRNAs in each sample. Each quantification was performed in duplicate and a negative control was included in all reactions.

3.12 Statistical Analysis

Statistical analysis of RNA quantifications and uptake levels of the engineered exosomes was performed using IBM®SPSS®Statistics software for Windows (Version 22.0). The $2^{-\Delta\Delta Ct}$ method (Livak method) and the t' Student test were used to evaluate the differences in the expression levels of the normalized mRNA and miRNA in study. The t' Student test was also used to evaluate the uptake differences of the fluorescent labeled exosomes by the renal cell lines in study.

4. Results

4.1 *In vitro* analysis of EGFR dysregulation in renal cell carcinoma

In order to evaluate EGFR dysregulation in the renal cell lines in study, mRNA expression levels of *EGFR* were assessed and the results were represented as expression fold-change in figure 7. According to the data, FG-2 cell line presented a 3.14 fold-increase of *EGFR* mRNA expression levels when compared to the HKC-8 cell line ($P<0.001$).

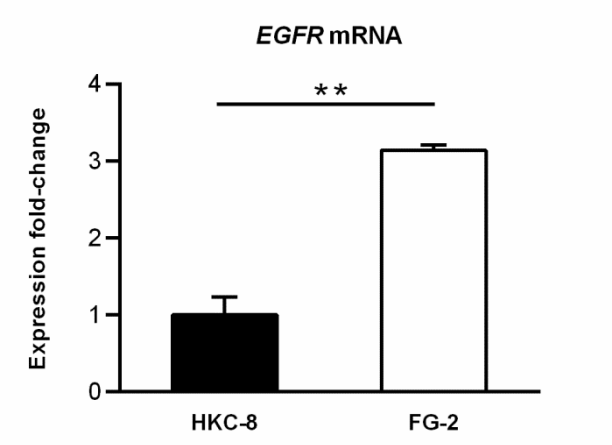

Figure 7 – Expression fold-change of *EGFR* mRNA levels between HKC-8 and FG-2 cell lines. Data is indicated as mean ± SEM. **, $P<0.001$.

Moreover, total EGFR protein levels were analyzed, as represented in figure 8 A, and a correlation between mRNA expression levels and protein levels was observed. Therefore, FG-2 cell line presented higher levels of total EGFR protein comparing to HKC-8 cell line. Furthermore, regarding EGFR phosphorylated levels at the site Y1068, data shows an overactivation of the receptor in FG-2 cells comparing to HKC-8 cells (Figure 8 B). In addition, according to the results represented in figure 8 C, ERK1/2 levels were similar in both cell lines. However, according to the data, the tumor renal cell line presented higher levels of phosphorylated ERK2, also known as p42, comparing to the normal renal cell line. Overall, these results indicate that EGFR is not only overexpressed but also overactivated in FG-2 cells which can lead to ERK2 overactivation.

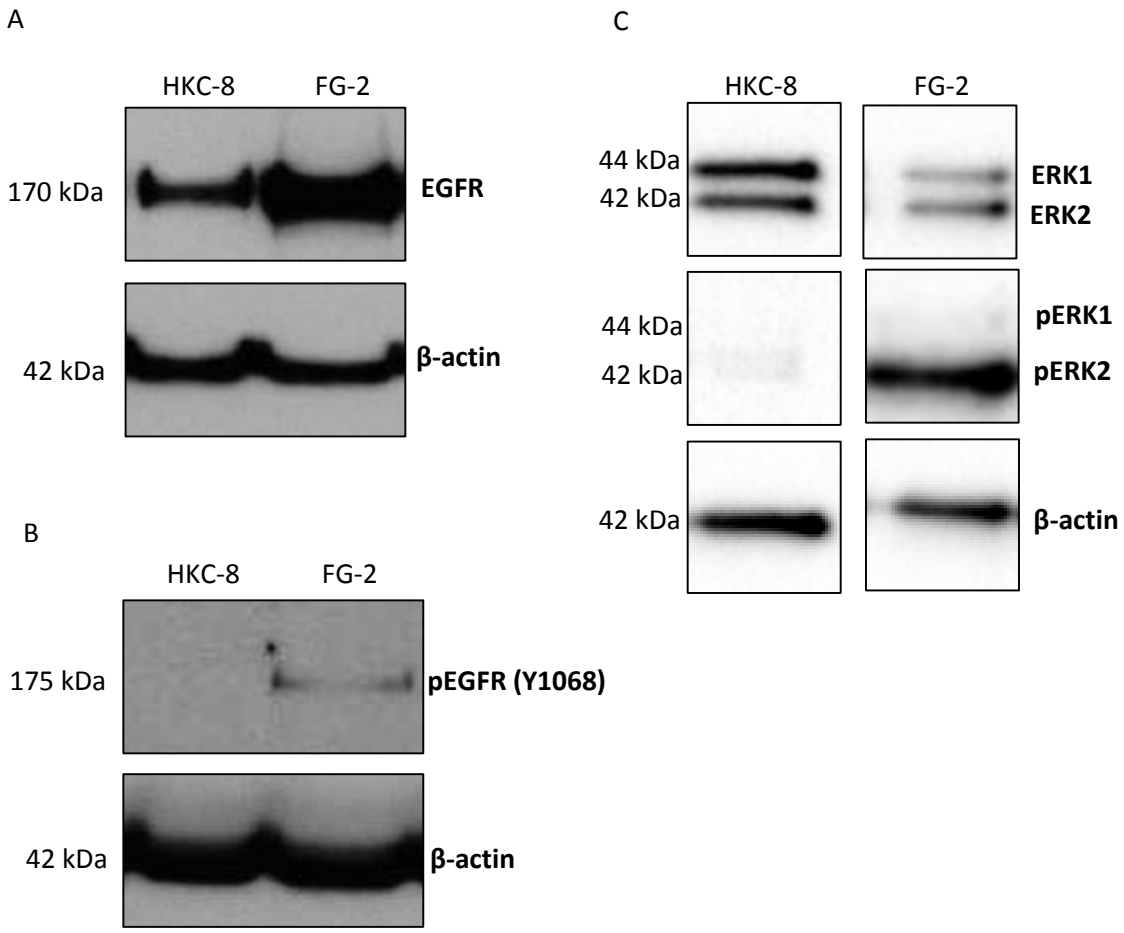

Figure 8 – Western-blot representing EGFR and ERK1/2 activation in HKC-8 and FG-2 cell lines. A) Total EGFR protein levels, B) Phosphorylated levels of EGFR at Y1068 site C) Total and phosphorylated levels of ERK1/2. The β-actin levels were used to normalize samples in all experiments.

4.2 Production of an EGFR-exosome targeted delivery system in HEK293T cell line

To generate GE11^{+} exosomes a plasmid was used in which the backbone vector was the pDisplay™ (Figure 9). Proteins expressed from pDisplay™ are fused at the N-terminus to the murine Ig κ-chain leader sequence, which directs the protein to the secretory pathway, and at the C-terminus to the PDGFR transmembrane domain, which anchors the protein to the plasma membrane, displaying the peptide of interest to the extracellular side.

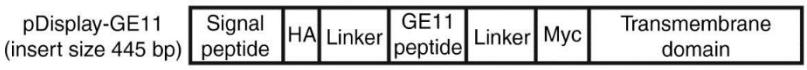

Figure 9 – Diagram of the GE11 construct. Signal peptide Igκ-chain leader sequence; HA, hemagglutinin epitope tag (YPYDVPDYA); Linker, (GGGGS)x3; Myc, Myc epitope tag (EEKLISEEDL); platelet-derived growth factor receptor (PDGFR) transmembrane domain.

HEK293T cells were transiently transfected with the plasmids of interest pDisplay or pDisplay-GE11. The plasmid mCherry was used as a transfection control (Figure 10).

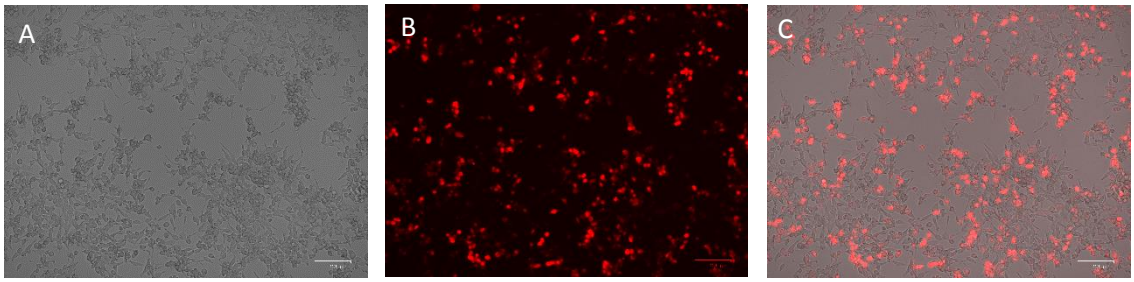

Figure 10 – Transfection control of HEK293T cells with mCherry plasmid. A) bright field B) red channel C) merge. ZOE Fluorescent Cell Imager (BIO-RAD®).

In order to evaluate the expression of the GE11 in HEK293T cells a western-blot was performed using anti-Myc or anti-HA antibodies (Figure 11 A and B, respectively). As expected, only HEK293T cells transfected with pDisplay-GE11 were positive for the Myc or HA tags.

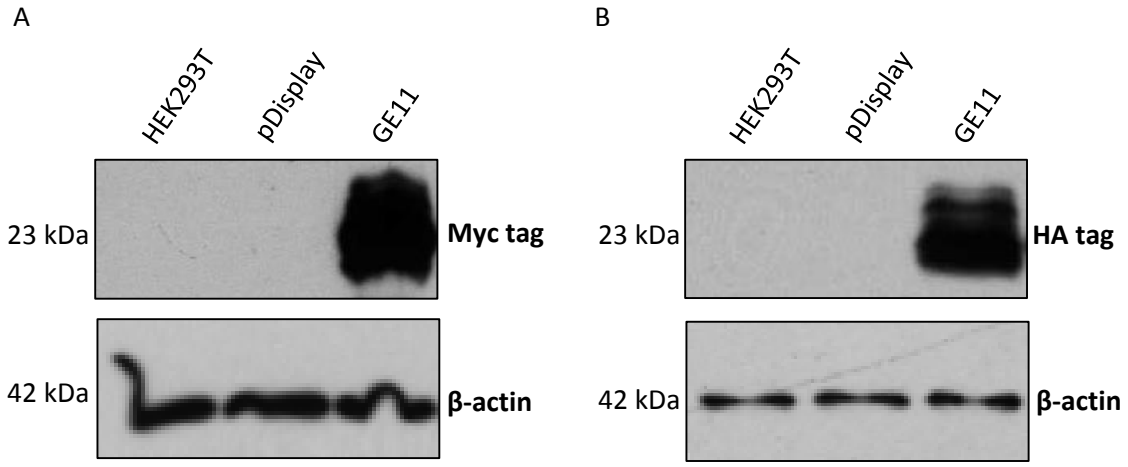

Figure 11 – Western-blot representing GE11 expression in HEK293T cells using A) anti-Myc antibody or B) anti-HA antibody. The β-actin levels were used to normalize samples in all experiments.

Regarding the immunofluorescence experiment, HEK293T cells were transfected with pDisplay-GE11 and as a negative control non-transfected HEK293T were used. Non-permeabillized cells were incubated either with anti-Myc or anti-HA antibodies (Figure 12 A and B, respectively). In accordance with the western-blot results, only HEK293T cells expressing GE11 were positive and results were similar for both antibodies.

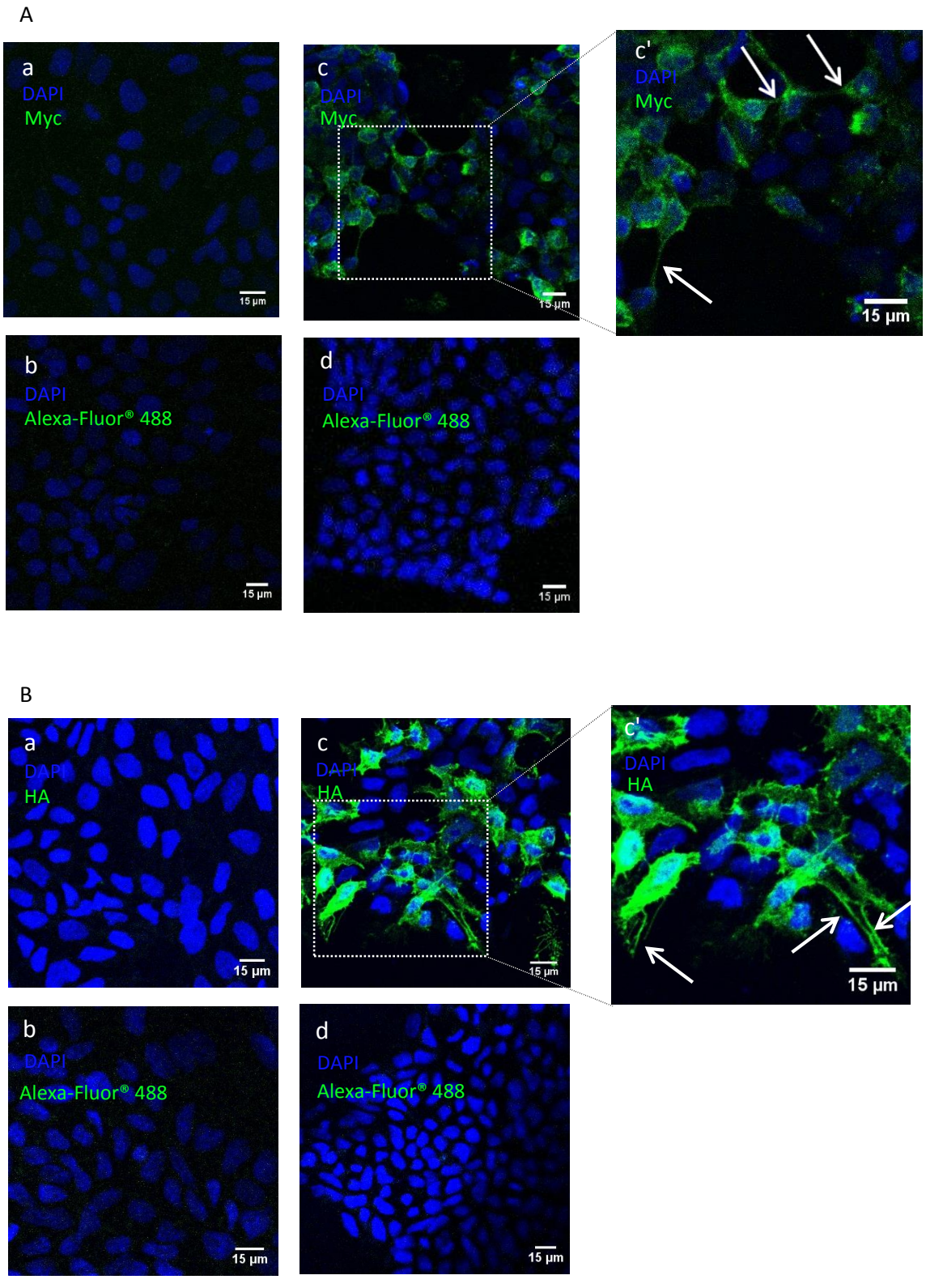

Figure 12 – Membrane-labeled HEK293T cells were detected when using A) anti-Myc or B) anti-HA antibodies, using confocal fluorescence microscopy. For both A) and B) diagrams: a) non-transfected HEK293T cells and c) HEK293T cells transfected with pDisplay-GE11, c') zoom in from image c) and arrows point to the membrane labeling. b) and d) represent the negative controls, respectively of a) and c), in which cells were incubated only with the secondary antibody, Alexa-Fluor® 488.

Since HEK293T cells were expressing and correctly directing the protein of interest to the cell membrane, exosomes were then isolated and purified for further analysis. In order to evaluate the expression of GE11 in HEK293T-derived exosomes a western-blot using anti-Myc or anti-HA antibodies was performed (Figure 13 A and B, respectively). According to the data, only exosomes derived from HEK293T cells transfected with pDisplay-GE11 were positive for the Myc or HA tags.

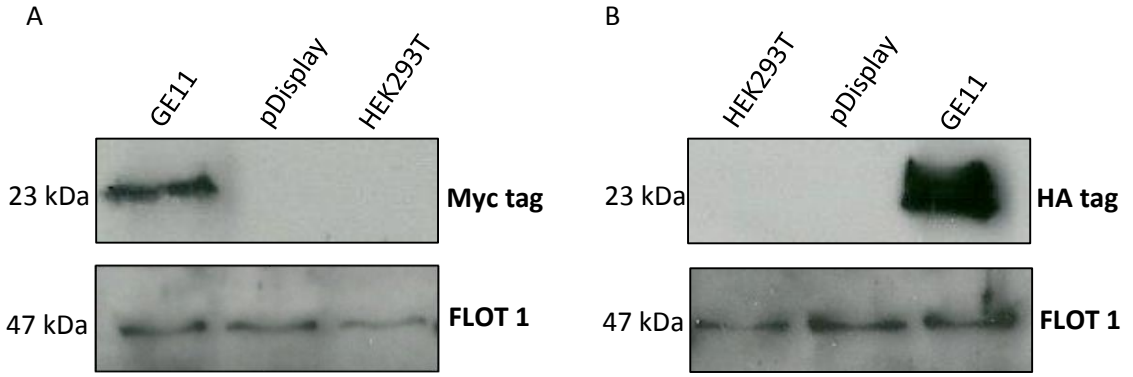

Figure 13 – Western-blot representing GE11 expression in HEK293T-derived exosomes using A) anti-Myc antibody or B) anti-HA antibody. The flotillin1 (FLOT1) levels were used to normalize samples in all experiments.

In order to assess if HEK293T-derived exosomes had the GE11 peptide displayed to the extracellular space transmission electron microscopy immunogold was performed. Samples were labeled either with anti-CD81 or anti-Myc antibodies and visualized using electron microscopy as represented in figure 14. According to the data, the method used to isolate and purify exosomes resulted in the visualization of bilayer nanoparticles raging in size approximately from 50 to 100 nm that were CD81 positive (Figure 14 A), which is consistent with exosomes characteristics. Exosomes derived from HEK293T cells previously transfected with pDisplay-GE11 were incubated with anti-Myc antibody and a membrane labeling was observed as represented in figure 14 C. As a negative control exosomes derived from non-transfected HEK293T were also probed with anti-Myc antibody and no labeling was observed as represented in figure 14 E.

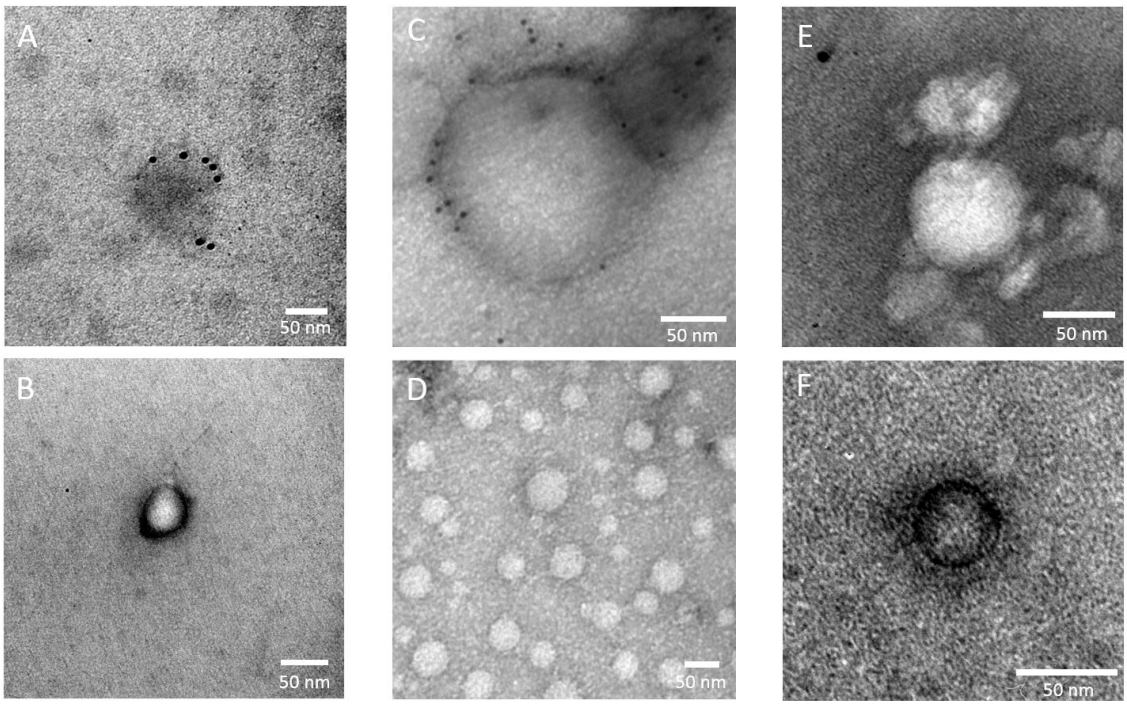

Figure 14 – Immunogold of HEK293T-derived exosomes. A) HEK293T exosomes incubated with CD81; B) HEK293T exosomes negative control: IgG anti-mouse gold; C) GE11^{+} exosomes incubated with anti-Myc; D) GE11^{+} exosomes incubated with IgG anti-mouse gold; E) non-transfected HEK293T-derived exosomes incubated with anti-c-Myc F) non-transfected HEK293T-derived exosomes incubated with IgG anti-mouse gold.

4.3 Analysis of the uptake by renal cell lines of the engineered exosomes

In figure 15 is represented total EGFR protein levels in the normal HK-2 and in the tumor FG-2 renal cell lines. In accordance with the results, FG-2 cells presented higher levels of total EGFR protein when compared to HK-2 cells. Hence, further studies were performed in order to evaluate the engineered exosomes uptake levels by HK-2 and FG-2 renal cell lines.

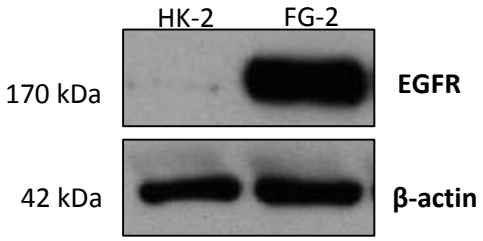

Figure 15 – Western-blot representing EGFR expression in HK-2 and FG-2 renal cell lines. The β-actin levels were used to normalize samples in all experiments.

Exosomes derived from HEK293T cells transfected with pDisplay (control condition) or pDisplay-GE11 were labeled with PKH67 dye (green) and added to cultures of FG-2 or HK-2 cells at either 4°C or 37°C, for 4 hours. As represented in figure 16, at 4°C there was almost complete abrogation of exosomes uptake. Regarding HK-2 cells there was 1.7% uptake in the control condition and 0.0% uptake of $GE11^+$ exosomes. Whilst in FG-2 cells there was 0.1% and 0.2% uptake in the control condition and after cells incubation with $GE11^+$ exosomes, respectively. Concerning the uptake analysis of the engineered exosomes by the renal cell lines at 37°C (Figure 16) a different pattern of uptake was observed, depending on the cell line in study.

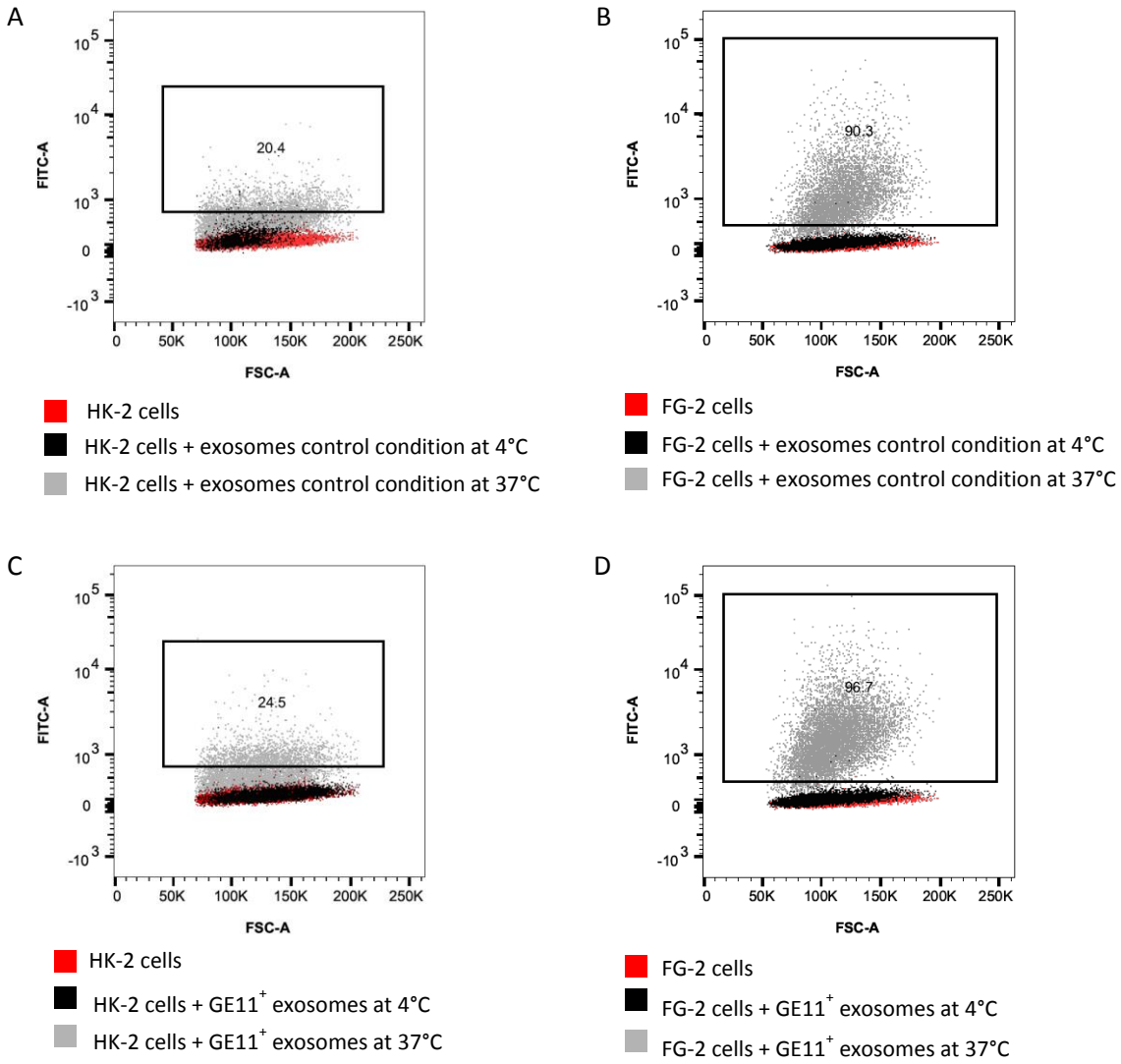

Figure 16 – Internalization levels of the fluorescent labeled engineered exosomes by HK-2 and FG-2 cell lines. A) and B) correspond to the uptake levels of exosomes derived from HEK293T cells transfected with pDisplay (control condition) after 4 hours incubation at 4°C or 37°C by HK-2 or FG-2 cell lines, respectively. C) and D) correspond to the uptake levels of $GE11^+$ exosomes after 4 hours incubation at 4°C or 37°C by HK-2 or FG-2 cell lines, respectively. Baseline settings were determined using a sample of each cell line that had not been treated. The percentages correspondent to the uptake levels of the engineered exosomes at 37°C are represented in each case. A representative experiment was used as an example.

Regarding HK-2 cells, despite the nature of the exosomes in study, the uptake levels remained similar (average levels were 19.7% and 22.2% for the control condition and GE11^{+} exosomes, respectively). Furthermore, as represented in figure 17 A, the median fluorescence intensity was the same whether looking at HK-2 cells incubation with exosomes derived from HEK293T cells transfected with pDisplay or pDisplay-GE11. Overall, no statistical significant differences were observed when comparing the uptake levels of GE11^{+} exosomes with the control condition in HK-2 cells. Conversely, for FG-2 cells, a statistical significant difference between the uptake of GE11^{+} exosomes (97.4%) and the control condition (91.8%) was observed (P<0.001). Additionally, as illustrated in figure 17 B, the median fluorescence intensity was approximately 1.4 times greater (P=0.001) when comparing the uptake of GE11^{+} exosomes with the control in FG-2 cells.

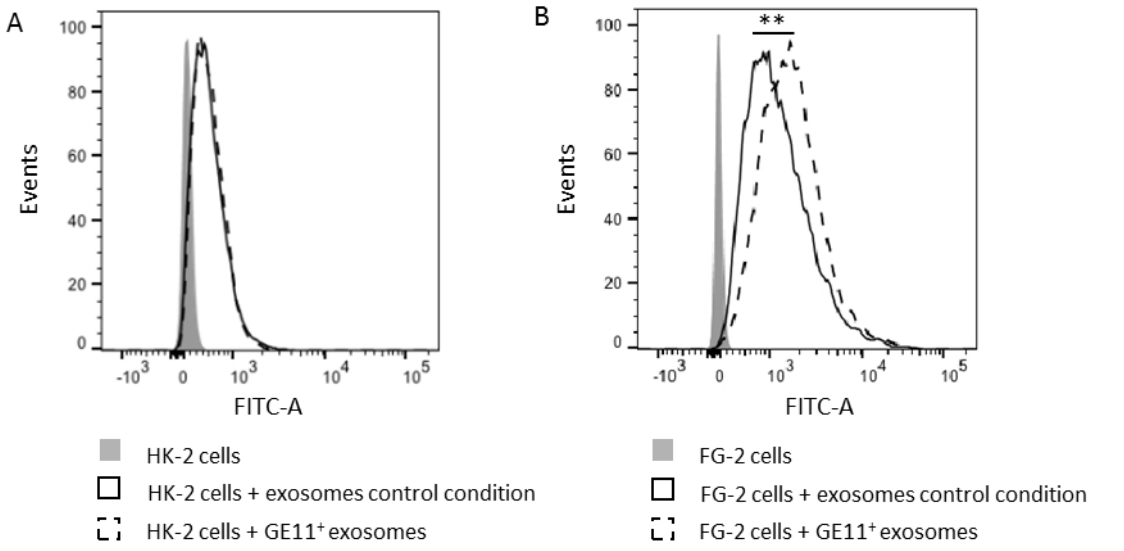

Figure 17 – Fluorescence intensity patterns of A) HK-2 cells and B) FG-2 cells after internalization of the engineered exosomes at 37°C for 4 hours. Events refer to cell counts that were normalized to mode. A representative experiment was used as an example.

Moreover, according to the results, there was a statistical significant difference between the uptake of the exosomes derived from HEK293T cells transfected with pDisplay, the control condition, by the HK-2 and FG-2 cell lines, 19.2% and 91.8%, respectively (P<0.001). Finally, when comparing the uptake levels of GE11^{+} exosomes from both renal cell lines in study, there was a significant increase in the uptake by tumor cells rather than by normal cells (FG-2: 97.4% and HK-2: 22.2%, P=0.001). Representative images of the engineered exosomes uptake by FG-2 cell line, 4 hours after incubation at 37°C are illustrated in figure 18.

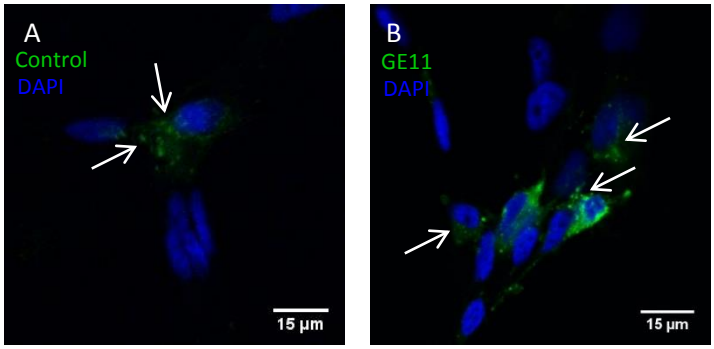

Figure 18 – Intracellular PKH67-labeled exosomes were detected in FG-2 cells using confocal fluorescence microscopy. Exosomes derived from HEK293T cells transfected with A) pDisplay (control) or B) pDisplay-GE11, are represented in green.

4.4 MiRNA-1233 and *TP53* mRNA functional relationship in renal cell carcinoma

In figure 19 A is represented the expression fold-change of miRNA-1233 levels in HKC-8 and FG-2 cell lines. According to the results, there was a 9 fold-increase in the intracellular levels of miRNA-1233 in FG-2 cell line when compared to HKC-8 cell line (*P*=0.007). In figure 19 B is represented the expression fold-change of *TP53* mRNA in both cell lines in study and there was a 0.54 fold-decrease in expression in FG-2 cells comparing to HKC-8 cells (*P*=0.016).

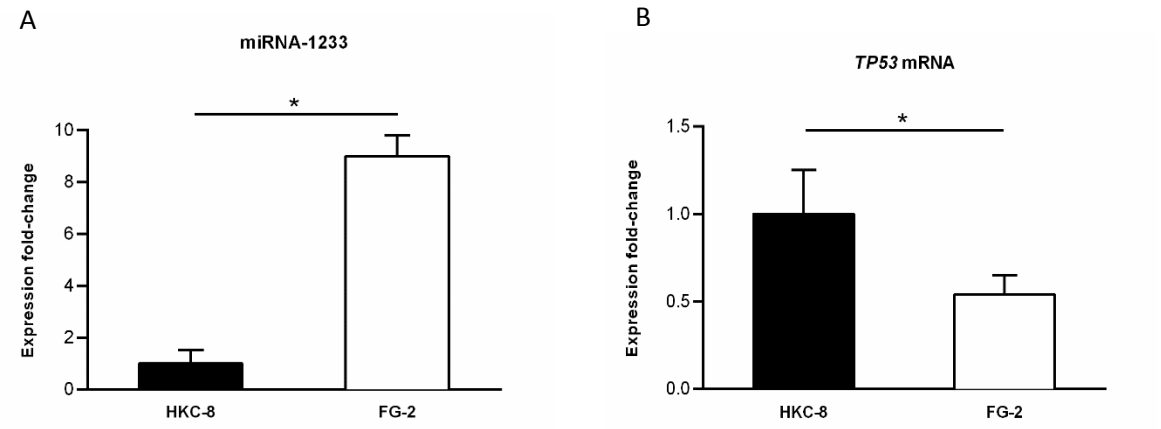

Figure 19 – MiRNA-1233 and *TP53* mRNA intracellular levels fold-change between HKC-8 and FG-2 renal cell lines. Data is indicated as mean ± SEM. *, *P*<0.05.

After miRNA-1233 and *TP53* mRNA expression levels characterization the functional relationship of these two molecules was assessed in the tumor renal cell line. Therefore, FG-2 cells were either transfected with a scramble sequence or with *miRVana®* miRNA-1233 mimic. 24 and 48h

after transfection, miRNA-1233 and *TP53* mRNA expression levels were assessed as shown in figure 20 A and B, respectively. The overexpression of miRNA-1233 was successfully achieved and according to data there was an 843 and a 77 fold-increase in expression 24 (P<0.001) and 48 hours (P=0.007) after transfection, respectively. Regarding *TP53* mRNA expression levels upon *miRVana®* miRNA-1233 mimic transfection, as represented in figure 20 there were no statistical significant differences observed.

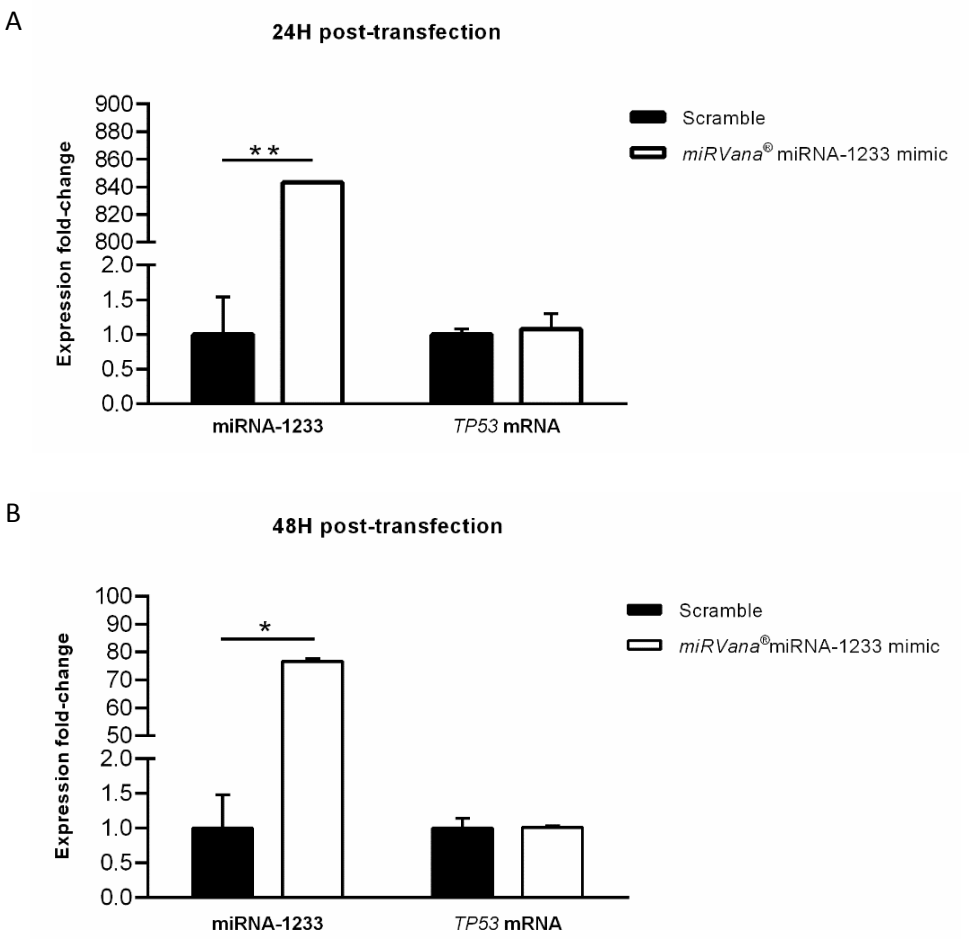

Figure 20 – MiRNA-1233 and *TP53* mRNA intracellular levels fold-change between FG-2 cell line A) 24 hours after and B) 48 hours after transfection with a scramble sequence or with *miRVana®* miRNA-1233 mimic. Data is indicated as mean ± SEM. *, P<0.05 and **, P<0.001.

5. Discussion

ccRCC is the most common and aggressive histological subtype of all renal cell carcinomas, and it is characterized by the frequent loss, mutation or epigenetic silencing of *VHL* gene [38, 39, 41]. *VHL* impairment induces a state of hypoxia in non-hypoxic conditions trough HIF accumulation and consequent transcriptional activation of various genes involved in hypoxia-adaptive responses [45]. One of those responses include increased expression of TGFα, an agonist to EGFR, able to enhance the receptors activity and therefore promote tumor growth [126]. The tumor renal cell line used in the present study is an *in vitro* model of a *VHL*-defective cell line thus, EGFR and ERK1/2 downstream signaling pathway were evaluated. According to our results, *EGFR* mRNA expression levels were higher in the metastatic ccRCC cell line when compared to normal proximal tubule epithelial renal cell line. Moreover, tumor cells presented higher protein levels of EGFR as well as higher phosphorylated levels at the site Y1068 of the receptor when compared to normal cells. Behind this pathway dysregulation may be several factors including the increased translational efficiency of *EGFR* mRNA induced by HIF2α and the delayed turnover of the activated EGFR in *VHL*-defective ccRCC cells [127]. The increased EGFR half-life can be directly related with the loss of pVHL that targets the phosphorylated protein to proteasome degradation or due to HIF accumulation that suppresses the activated EGFR lysosomal-mediated degradation [55, 56]. After EGFR activation via the phosphotyrosine residue Y1068, Grb2 adaptor protein is recruited and MAPK/ERK1/2 downstream signaling is activated [50, 128]. Indeed, our data also suggested an overactivation of MAPK pathway since higher levels of ERK2 phosphorylated form were detected in tumor cells comparing to normal cells. Once activated, ERK is able to translocate to the nucleus and activate various transcription factors, including Fos and STAT3 [129]. Ultimately, these activated transcription factors modify gene expression profiles that regulate cell proliferation and survival, resulting in cell growth induction [129]. Moreover, it has been shown that ERK1/2-MAPK can increase HIF1 transcription activity directly by phosphorylating HIF1α or by phosphorylating the p300/CBP co-activator [130, 131]. Taken together, *VHL* and HIF dysregulation, higher levels of TGFα, EGFR overexpression and downstream signaling activation contribute to a positive feedback loop that sustains an autocrine solicitation of proliferation and survival, contributing to tumor growth. In fact, recent studies have demonstrated that higher EGFR expression levels have been correlated with higher tumor grade and shorter survival in ccRCC patients [55, 132, 133]. Overall, these findings highlight the importance of EGFR in ccRCC development and progression.

Approximately one-third of all patients are diagnosed with metastatic disease due to the fact that ccRCC remains asymptomatic until late stages of the disease [21, 25]. Furthermore, this tumor is notoriously resistant to chemotherapy, radiotherapy and to the targeted therapies today applied in the clinic [28, 33-35]. Therefore, there is the need to develop new therapeutic strategies that overcome these therapy limitations. EGFR dysregulation could be the base of new targeted therapies development. It has been demonstrated that engineered exosomes that express a certain ligand on their surface will bind to a targeted cells receptor and deliver their content to the recipient cell [117]. The production of $GE11^+$ exosomes is one good example. In the course of our study, an exosome-based delivery system was developed in which the peptide GE11 was facing to the extracellular space. It has already been proven that the GE11 peptide is able to bind specifically to EGFR, allowing an EGFR-dependent uptake by the targeted cells [121]. Ohno and colleagues demonstrated that the $GE11^+$ exosomes internalization levels correlated with EGFR expression levels in different breast cancer cell lines. Additionally, after *EGFR* knock-down in a breast cancer cell line, a decrease in $GE11^+$ exosomes internalization levels was observed [121]. More importantly, they demonstrated that a $GE11^+$ exosomes-based delivery system was viable *in vivo*, since $GE11^+$ exosomes loaded with miRNA let-7a significantly suppressed tumor growth in an orthotropic breast cancer model in $RAG2^{-/-}$ mice. Concerning our experiments, the engineered exosomes were incubated with either the normal or tumor renal cell lines at 4°C. At this condition, the exosomes uptake was almost inexistent, suggesting that cells had to be biologically active in order for this process to occur. In addition, since low temperatures decrease membrane fluidity, at 4°C cell membranes become more rigid, possibly preventing exosomes uptake [134]. These results are in accordance with others that also performed incubation at 4°C of cells treated with fluorescently labeled exosomes [134-136]. On the other hand, when cells were incubated at 37°C we were able to see exosomes accumulating inside of the cells. Several exosomes were located in the perinuclear region, suggesting that some might have followed the endocytic pathway [136]. Furthermore, when analyzing the uptake levels of the engineered exosomes by the tumor cell line we could see an increase of approximately 6% in the uptake of $GE11^+$ exosomes comparing to exosomes derived from HEK293T cells transfected with pDisplay, the control condition. The difference between the engineered exosomes uptake is due to an EGFR-dependent mechanism. We might have not seen a major increase because we were close to the saturation levels of the flow citometry, since both values were above 90%. Therefore, in future studies a scale-down of the amount of exosomes used to incubate with cells could result in greater exosomes uptake variations. Nonetheless, by analyzing the fluorescence intensity from the tumor cell line we observed that cells that

internalized GE11$^+$ exosomes had a higher fluorescence intensity median comparing to the control condition. This could indicate that not only the percentage of cells that internalized the GE11$^+$ exosomes was higher but there were more exosomes per cell, thus enforcing the uptake differences results and suggesting a supplementary uptake, EGFR-dependent. Regarding the uptake levels of the engineered exosomes by the normal renal cell line there were no significant differences and the overall uptake levels went around 20%. These data is in accordance with the total EGFR protein levels that were very low in normal renal cells, comparing to tumor renal cells. Hence, in the normal cells no significant increase in the uptake of GE11$^+$ exosomes comparing to the control was expected. Additionally, there were no statistical significant differences between the fluorescence intensity median of HK-2 cells that had internalized GE11$^+$ exosomes comparing with the control, which correlated with the overall percentage levels of internalization. Moreover, these data suggests that the non-EGFR dependent exosomes uptake levels vary between normal and tumor renal cell lines. Regarding the tumor cell line, there was approximately a 92% of exosomes uptake that was not EGFR-dependent whereas, for the normal renal cell line this value dropped to around 20%. The intrinsic capacity of each cell type to internalize exosomes might be different. Recent studies performed by Czernek and colleagues showed that the differentiation status of myeloid cells influences the efficiency of exosomes uptake since macrophages and mature dendritic cells presented higher exosomes uptake comparing to monocytes or immature dendritic cells [137]. Another study conducted by Hazan-Halevy demonstrated that Mantle cell lymphoma (MCL)-derived exosomes were preferentially taken up by MCL cells, when co-cultured with other cell types. However, the specificity of exosomes uptake by different target cells remains largely unknown and further studies are needed in order to elucidate the underlying mechanisms of these processes. Finally, when comparing GE11$^+$ exosomes uptake levels between normal and tumor renal cell lines a significant difference was observed. Approximately, 22% of the normal renal cells internalized the engineered exosomes, while in the case of the tumor cells the percentage rose to 97%. Therefore, according to our data, the use of GE11$^+$ exosomes as a new delivery system in ccRCC is very promising.

Ultimately, engineered extracellular vesicles can be loaded with RNAi-based drugs, for instance miRNA mimics or antagonists, and allow a more specific delivery of their content. It has been demonstrated that miRNAs are aberrantly expressed in cancer and that regulate different signaling pathways associated with tumor progression [65]. Increasing evidence supports the involvement of miRNAs in the regulation of VHL/HIF axis that ultimately affects EGFR-related pathways [138]. For instance, miRNA-92 is overexpressed in chronic lymphocytic leukemia B

cells and targets *VHL* mRNA, downregulating its protein levels and promoting HIF/VEGF axis activation [139]. Moreover, this miRNA correlated negatively with both *VHL* mRNA and pVHL levels in ccRCC tumor samples [140]. These studies demonstrate the potential role of certain miRNAs in central pathways regulation such as the VHL/HIF one. Regarding this axis dysregulation it is known that HIF can be directly regulated by p53 through MDM2-mediated ubiquitination and proteasome degradation [70]. Furthermore, Wulfken and colleagues demonstrated that miRNA-1233 could target p53, based on a bioinformatics approach [72]. Thus, we hypothesized that miRNA-1233 could regulate p53 and consequently HIF responses related to hypoxia, making him a promising drug target candidate. Indeed, studies developed in our research group demonstrated the upregulation of miRNA-1233 in plasma samples of ccRCC patients when compared to healthy individuals [71]. In accordance with these findings, the tumor renal cell line used in this study presented higher expression levels of miRNA-1233 when compared to the normal renal cell line. Besides, *TP53* mRNA expression levels were decreased in tumor cells which reflects a negative correlation between these two molecules, enforcing our hypothesis. However, when the tumor cell line was transfected with a miRNA-1233 mimic, no differences were observed in *TP53* mRNA levels. However, this result does not discard the possibility of *TP53 mRNA* being a target of miRNA-1233. It is known that miRNAs can induce gene repression mainly through two different ways: 1) at the level of mRNA stability by miRNA-mediated mRNA decay or by sequestration of target mRNAs in P-bodies (cytoplasmic structures in which the mRNA degradation machinery is enriched) or 2) at the level of mRNA translation including repression of initiation and/or elongation, ribosome drop-off, and nascent polypeptide degradation [141, 142]. One frequent mechanism of miRNAs regulation of gene expression is by base pairing imperfectly to the 3'UTR sequences of target mRNAs, inhibiting protein synthesis, but maintaining the mRNA levels [143]. Therefore, further studies would be necessary to completely clarify miRNA-1233 and p53 interaction in order to elucidate its role in the hypoxia-state maintenance in ccRCC. For instance, by transfecting the tumor renal cell line with a miRNA-1233 mimic or inhibitor and evaluating p53 protein levels by western-blot. Nonetheless, miRNA-1233 dysregulation and its potential oncogenic role in ccRCC makes it a new promising candidate for drug targeting. MiRNA-1233 inhibition could be achieved by various synthetic modified antisense oligonucleotides such as AMOs, LNA anti-miRNAs, miRNA sponges, and others [118]. LNA anti-miRNAs are of special interest due to their ability to knock-down miRNAs expression *in vivo* [144]. Although miRNA-based therapeutics appears very promising, there is a major hurdle associated with an effective method of delivery. The presence of serum and cellular nucleases make these molecules very unstable and thus, unfeasible to use on their own [145, 146].

Additionally, their small size and negative charge interfere with their ability to cross cell membrane [146]. Hence, RNAi-based therapeutics requires a targeted delivery vehicle for sufficient tissue specificity and cellular uptake. Amongst all the possibilities exosomes have received especial attention due to being natural occurring RNA nanocarriers [81, 82, 118]. The combination of RNAi-based drugs with engineered exosomes as a new strategy for ccRCC treatment is very promising. Additional studies should be performed in order to validate the potential use of $GE11^+$ exosomes as a drug delivery vehicle not only in the ccRCC tumor model but also in other EGFR-overexpressing tumors. Regarding the use of engineered exosomes in the clinic, the insufficient understanding of exosomes role in normal and pathological conditions makes it difficult to predict therapeutic effectiveness and long-term safety [147]. More studies are necessary in order to clarify exosomes *in vivo* trafficking, their biological fate and impact on targeted organ [76, 148].

Overall, $GE11^+$ exosomes can be used and loaded with a miRNA-1233 antagonist and potentially affect tumor development, becoming a novel therapeutic approach in ccRCC. This strategy can be applied in different disease contexts due to the versatility of engineered exosomes and its loading with a wide range of RNAi-based drugs, allowing a potent drug targeted effect.

6. Conclusion and Future Perspectives

VHL impairment and consequent HIF accumulation is a crucial event in ccRCC initiation, responsible for several pathways dysregulation including EGFR activation through TGFα production. In the present study, *in vitro* experiments demonstrated EGFR overexpression and overactivation in a metastatic ccRCC cell line. Additionally, downstream signaling MAPK/ERK was overactivated since the tumor renal cell line presented higher levels of ERK2 phosphorylated form comparing to the normal renal cell line. EGFR downstream signaling is associated with various cell signaling pathways that regulate proliferation and survival. Through EGFR dysregulation tumor cells become self-sufficient in growth signals which ultimately leads to malignancy progression. Although this study has given very useful insights about EGFR importance in ccRCC tumor model, in future studies these experiments should be replicated in other ccRCC-derived cell lines that could reflect different ccRCC stages of development, since we only had the opportunity to use a metastatic ccRCC cell line. Besides, it would also be very interesting to analyze other downstream signaling cascades activation, such as PI3K/AKT and STAT3 and evaluate if EGFR inhibition would reflect in a downstream inhibition of those pathways. Meaning that those cascades activation would arise primarily from EGFR activation, once again demonstrating the central role of EGFR in ccRCC progression. Since this tumor is resistant to chemotherapy, radiotherapy and to the targeted therapies today applied in the clinic, there is the need to develop new therapeutic strategies that overcome these therapy limitations. EGFR dysregulation could be the base of new targeted therapies development. Recently, exosomes have emerged as new therapeutic tools, since they can be engineered to express certain cell type-specific ligands on their surface, like the GE11 synthetic peptide, an EGFR ligand. In the present study, exosomes derived from HEK293T cells were engineered in order to express the GE11 peptide on their surface and incubated with either normal or tumor renal cell lines in vitro at 4°C or 37°C. After incubation at 4°C there was almost complete abrogation of exosomes internalization by both renal cell lines suggesting that cells had to be biologically active in order for this process to occur. Regarding cells incubation at 37°C, those experiments demonstrated that a higher percentage of metastatic ccRCC cells internalized $GE11^+$ exosomes comparing to exosomes derived from HEK293T cells transfected with pDisplay, the control condition. Besides, the number of $GE11^+$ exosomes per cell was also higher, evidencing those uptake differences. Regarding the normal renal cell line, as expected, those differences were not observed since their EGFR expression levels were lower when compared to the tumor renal cell line. Overall, there was a significant difference between the $GE11^+$

exosomes uptake by the normal and tumor renal cell lines. However, uptake differences of $GE11^+$ exosomes were not exclusively due to different EGFR expression levels between both renal cell lines, since there was a significant difference concerning the uptake levels in the control condition. Nevertheless, these findings suggest that using $GE11^+$ exosomes as a new delivery system in ccRCC is very promising. Future studies should include a wider range of ccRCC-derived cell lines that reflect different EGFR expression status and also additional normal renal cell lines in order to evaluate the reproducibility of this delivery system. Engineered exosomes are promising delivery vehicles of RNAi-based drugs due to their high stability in circulation and targeted cell type-specificity. Increasing evidence supports the involvement of miRNAs in VHL/HIF axis regulation as it was hypothesized for miRNA-1233. Despite initial findings that miRNA-1233 was upregulated, whereas *TP53* mRNA was downregulated in the tumor renal cell line comparing to the normal renal cell line, we were not able to find a direct correlation between miRNA-1233 and *TP53* mRNA. Therefore, in order to evaluate the potential role of miRNA-1233 in HIF/EGFR axis regulation, further studies should focus on p53 protein levels assessment through a western-blot, upon tumor cells transfection with a miRNA-1233 mimic or inhibitor. Furthermore, if this functional relationship was established, it would also be interesting to analyze the influence of miRNA-1233 levels variation in HIF and EGFR expression, once again possibly through western-blot analysis. Nonetheless, miRNA-1233 high expression levels detected in the metastatic ccRCC cell line, in addition to previous findings in plasma samples of ccRCC patients, demonstrate the potential importance of this miRNA in ccRCC development. Therefore, future studies should center in its *in vitro* inhibition and evaluation of diverse biological processes such as cell proliferation, death, migration, and invasion, since it appears a very promising candidate for drug targeting. Ultimately, future experiments should include *in vivo* validation of this delivery system, by using $GE11^+$ exosomes loaded with a miRNA-1233 antagonist in a ccRCC mice model. These experiments would reveal the potential use of an exosome and RNAi-based therapy in ccRCC treatment.

7. References

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8. Attachments

Review

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Pharmacogenomics

miRNAs: mediators of ErbB family targeted therapy resistance

The ErbB/HER tyrosine kinase receptors family plays a key regulatory role in different cellular processes by activating several signaling pathways. In different tumor types, mutations or overexpression of the ErbB family members are a common feature, which led to the development of targeted therapies against this receptors. Although with this kind of treatment we are heading to a more personalized medicine, the development of acquired resistance is still an issue, therefore, several studies focused on discovering the mechanisms behind it. More recently, miRNAs have been described as important mediators of acquired resistance, specifically, acquired resistance to ErbB family targeted therapies. Ultimately, miRNA-based therapeutics using exosomes as a drug delivery model can revolutionize today's approach of cancer treatment.

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Keywords: acquired resistance • ErbB family • exosomes • miRNAs • targeted therapies

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Background

miRNAs

miRNAs are a class of noncoding RNAs (19–25 nucleotides in length) that control gene expression by either degrading or blocking translation of mRNAs, a process that depends on the miRNA and the respective mRNA target degree of complementarity [1]. miRNA biogenesis is a multiphase process which initiates at the nucleus with the transcription of a primary RNA, the pri-miRNA, by RNA polymerase II [2]. Following transcription, pri-miRNA is processed by Drosha, a RNase III endonuclease, alongside with cofactor DGCR8, creating a pre-miRNA [3]. This precursor ultimately is exported to the cytoplasm by Exportin 5 where is processed by another RNase, Dicer, leading to the production of a mature 22 base pairs miRNA duplex [2]. The mature miRNA enters the RNA-induced silencing complex (RISC), whose main components are TRBP, AGO1–4, GEMIN3 and GEMIN4 becoming, ultimately, a functional miRNA [4]. This complex binds to the target mRNA at 3′UTR region by complementarity leading to gene silencing [5].

A miRNA is not specific for a certain mRNA, it can regulate up to 100 different mRNAs and is also described that more than 10,000 mRNAs seem to be regulated by miRNAs [6]. Thus, changes in the miRNA processing and expression patterns could be associated with different pathologies, including cancer, suggesting that miRNAs are involved in many cellular function disorders, which includes carcinogenesis [7].

After the discovery of miRNA-15a and miRNA-16-1 as the first miRNAs with tumor suppressor functions in chronic lymphocytic leukemia in 2002, many miRNAs have been described as mediators of cancer-related signaling pathways, regulating proliferation, apoptosis, angiogenesis and even epithelial–mesenchymal transition (EMT), a key step for the metastatic process [8,9]. Since miRNAs are associated with different biological processes, they have been described

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to act as oncogenes, tumor suppressors or even modulators of cancer stem cells and metastasis formation. OncomiRNAs are, usually, overexpressed in cancer since they are known to downregulate tumor suppressor genes and tumor suppressor miRNAs are responsible for downregulating oncogenes, so are mostly underexpressed in malign neoplasms [10,11]. However, this dichotomous approach may have its limitations. For example, miRNA-17 is associated, in B-cell lymphoma, with faster tumor development, while it can suppress cancer growth by downregulating AIB1 expression in breast cancer [12,13]. Therefore, we have to take in consideration the fact that miRNAs may act in a tissue-specific manner such that a single miRNA can be either an oncomiRNA or a tumor suppressor miRNA.

Regarding the miRNAs role in cellular processes such as proliferation, apoptosis or angiogenesis, it is important to take into account the effect that changes in miRNA levels may have in treatment response, since targeted therapies are used for specific proteins and signal pathways related with this biological processes [8,9].

Cancer treatment

Chemotherapy, along with surgery and radiotherapy, has been a crucial approach for cancer treatment. These different types of treatment can be used alone or in different combinations, and either simultaneously or sequentially. However, chemotherapy-/radiotherapy-induced cell damage occurs preferentially but not exclusively in cancer cells, causing many side-effects [14]. For this reason, nowadays, a big effort is being made in order to improve a personalized medicine that focus on the discovery and development of molecular targeted drugs that take advantage of genetic addictions, dependencies and vulnerabilities of cancer cells. This type of methodology would be more specific than previous described approaches by minimizing side effects on normal cells [15]. Additionally, different high-throughput technologies, still under development, like genome sequencing and various kinds of microarrays, will allow the knowledge of the genetic, epigenetic and proteomic background, from each individual and tumor, which ultimately will lead to a more personalized treatment [16].

The concept of targeting a pathogenic driver abnormality using a small molecule was first validated in 1988 by the successful treatment of patients with acute promyelocytic leukemia harboring translocations in the *RARα* gene with all-trans retinoic acid [17]. Additionally, the use of imatinib, a BCR-ABL inhibitor, as a chronic myeloid leukemia treatment in 1996, marked the era of the design of small therapeutic molecules applied in cancer treatment [18]. The 5-year estimated overall survival rate for patients with this malignancy, characterized by the BCR-ABL translocation, was 89% when imatinib was used as initial therapy [19].

Even with all this development and improvement in cancer therapy, resistance to treatment still exists. Therapy failure is often due to development of drug resistance that may be inherent in a subpopulation of heterogeneous cancer cells or acquired subsequent to treatment [20]. A well-characterized resistance mechanism is related with the activity of ABC transporters. ABC transporters are transmembrane proteins responsible for the transport of a wide variety of substrates across cellular membranes, including hydrophobic drugs and antibiotic [21,22]. Overexpression of these proteins can be associated with reduced drug uptake, increased drug efflux and lead to lower drug efficacy and possibly to acquire drug resistance due to low drug levels in the cytoplasm [22]. The major members of the ABC transporters associated with multidrug resistance in cancer cells are ABCB1/MDR1, ABCCs (MRPs) and ABCG2 (BCRP/MXR/ABCP) [23]. However, several ABC transporters have been identified as transporters of cancer chemotherapeutics agents, acquired chemotherapy drug resistance can occur at many levels, modulated either by genetic or epigenetic factors. In fact, recent data demonstrate that the activity of certain miRNAs might be altered in order to achieve resistance to chemotherapy [24]. In the same line of thought, miRNAs can be as well linked to acquired resistance in molecular targeted therapy in several malignancy treatments. Latest evidences support this idea as it will be described next.

This review focus in the role of miRNAs as mediators of acquired resistance in ErbB family targeted therapies since this family plays a key regulatory role in nearly every aspect of cell biology. The ErbB/HER family contains four tyrosine kinase receptors, the epidermal growth factor receptor (EGFR/ErbB/Her1), Her2 (Neu, ErbB2), Her3 (ErbB3) and Her4 (ErbB4) [25]. Different factors as the identity of the ligand and oligomer composition of the receptor determine the specificity and potency of intracellular signals [26]. Downstream ErbB signaling includes phosphatidylinositol 3-kinase/Akt (PKB) pathway, the Ras/Raf/MEK/ERK 1/2 pathway and the phospholipase C (PLC-γ) pathway [27]. All of these pathways are interconnected and overlapping [26]. Overall, they regulate apoptosis, cell cycle progression, cytoskeletal rearrangement, differentiation, development, immune response, nervous system function and transcription [28]. Mutations or increased expression of ErbB family members occur in several malignancies [26,29]. For instance, ErbB1 overexpression occurs in head and neck, breast, bladder, prostate, kidney,

non-small-cell lung cancer (NSCLC) and glioma tumors, while mutations leading to a constitutively active receptor occur in glioma, lung, ovary and breast cancer. Overexpression of ErbB2 is frequent in breast, lung, pancreatic, colon, esophagus, endometrium and cervix cancer, whereas ErbB3 is overexpressed in oral squamous cell cancer [26,30].

Several targeted drugs have been developed against these protein kinases, however, cancers submitted to targeted therapy eventually become resistant [31,32]. miRNAs may be a way not only to unveil resistance mechanisms (Table 1) but also, if used as a treatment option, to overcome targeted therapy limitations.

miRNAs & targeted therapy resistance

ErbB targeted therapies resistance in cancer

Head & neck cancer

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common form of cancer worldwide with 650,000 new cases each year [45]. Palliative chemotherapy and the EGFR inhibitor, cetuximab, constitute the backbone of treatment for patients with HNSCC [46]. However, many patients with HNSCC tumors do not respond to EGFR-targeting therapies [47].

Hatakeyama and coworkers demonstrated that one potential mechanism of acquired resistance to cetuximab in HNSCC involves the increased expression of HB-EGF that is regulated by miRNA-212. HB-EGF is known to bind both EGFR and HER4 and to induce EMT, enhance metastasis and modulate chemotherapy resistance [48–50]. miRNA-212 showed a 27-fold decrease in 1Cc8 cetuximab-resistant cell line relative to SCC1 cetuximab-sensitive cell line. Expression levels of HB-EGF and miRNA-212 were also examined in 32 additional HNSCC cell lines and keratinocyte cell line, demonstrating the inverse correlation of this two parameters. Increased expression of HB-EGF regulated by miRNA-212 and activation of receptor kinases other than EGFR, like HER3 and MET, and subsequent activation of AKT, were observed in 1Cc8 cell line, and may play an important role in acquired resistance to cetuximab [33].

Lung cancer

Lung cancer is the leading cause of cancer related death worldwide [51]. Of all lung cancer cases, approximately 80–85% correspond to NSCLC [52]. One of the main issues regarding the therapeutic approach using chemotherapy or EGFR-tyrosine kinase inhibitors (TKIs) in NSCLC is the acquired resistance that develops short after treatment [35,53]. In fact, the role of miRNAs, more specifically miRNA-21, has already been reported in NSCLC as a modulator of chemotherapy sensitivity [54]. Additionally, Shen *et al.* stated that this same miRNA correlated with PTEN levels (one of its most important targets) modulates gefitinib resistance in the same tumor model [55]. They ana-

Table 1. Summary of the miRNAs involved in the acquired resistance to ErbB family targeted therapies by cancer type.

Cancer	Targeted Therapy	miRNA involved	Ref.
Head and neck cancer	Cetuximab	↓ miRNA-212	[33]
Lung cancer	Erlotinib	↑ miRNA-200 family	[34]
	Gefitinib	↑ miRNA-21	[35]
		↑ miRNA-30c, ↓ miRNA-103, ↓ miRNA-203, ↑ miRNA-221 and ↑ miRNA-222	[36]
		↑ miRNA-214	[37]
		↑ miRNA-374a and ↓ miRNA-548b	[38]
Gastric cancer	Trastuzumab	↑ miRNA-21	[39]
Breast cancer	Trastuzumab	↑ miRNA-21	[40]
		↑ miRNA-221	[41]
		↓ miRNA-375	[42]
	Lapatinib	↓ miRNA-630	[43]
	Neratinib		
	Afatinib		
Coloretal cancer	Cetuximab	↓ miRNA-let7b, ↓ miRNA-let7e and ↑ miRNA-17	[44]

↑: Upregulation; ↓: Downregulation.

lyzed the expression of miRNA-21 and PTEN protein in tumor tissues from NSCLC patients, comparing cancer tumor specimens with adjacent normal tissues. A significantly higher expression of miRNA-21 and a reduction in PTEN protein levels was found in tumor tissues, demonstrating a negative correlation. High miRNA-21/low PTEN expression levels indicated a poor TKI clinical response and shorter overall survival in NSCLC patients. In order to test the effect of high miRNA-21/low PTEN expression on modulation of TKI sensitivity, a PC-9 TKI-sensitive cell line and a gefitinib-resistant cell line PC-9/GR were used. *In vitro* assays showed that miRNA-21 was upregulated concomitantly to downregulation of PTEN in PC-9/GR cells. Moreover, overexpression of miRNA-21 significantly decreased gefitinib sensitivity by down-regulating PTEN expression and activating AKT and ERK pathways in PC-9 cells. Whereas, miRNA-21 knockdown dramatically restored gefitinib sensitivity of PC-9/GR cells by up-regulation of PTEN expression and inactivation of AKT and ERK pathways, both *in vivo* and *in vitro* [56].

Another study performed by Izumchenko and coworkers demonstrated that TGFβ–miRNA200–MIG6 pathway coordinates the EMT-associated kinase switch that induces resistance to EGFR inhibitors [34]. They evaluated pairs of cancer cell lines with wild-type *EGFR* that were either sensitive (epithelial-like) or resistant (mesenchymal-like) to erlotinib, an EGFR TKI. Treatment of erlotinib-sensitive cell lines with TGFβ resulted in complete EMT. Cells with induced mesenchymal phenotype, had both total EGFR and phospho-EGFR reduced and elevated expression of MIG6, acquiring a relative resistance to erlotinib, associated with a significant increase in AKT activity, due to higher levels of phospho IGFR, PDGFR, FGFR and FAK kinases [36,57]. Concurrently, expression levels of miRNA200 family decreased significantly. During TGFβ-mediated EMT, inhibition of the miRNAs 200 family results in upregulated expression of the MIG6, a negative regulator of EGFR. The MIG6-mediated reduction of EGFR occurs concomitantly with a TGFβ-induced EMT-associated kinase switch of tumor cells to an AKT-activated EGFR-independent state. The expression levels MIG6 (mRNA)/miRNA200 ratio were inversely correlated with EMT and resistance to erlotinib, in both *in vitro* and *in vivo* models. Demonstrating that TGF-β–miRNA200–MIG6 network orchestrates the EMT-associated kinase switch that induces resistance to EGFR inhibitors [34].

Studies in NSCLC also revealed an involvement of MET oncogene in TKIs resistance [37,58]. Garofalo and coworkers demonstrated that MET and EGFR-related miRNAs had a significant role in gefitinib resistance on NSCLC cell lines and *in vivo* models. NSCLC gefitinib-resistant cell lines, Calu-1 and A549, did not revealed an expected miRNA-30b-c and miRNA-221/-222 down-regulation and consequent increase in BIM and APAF-1 protein levels after treatment. miRNA-30b-c and miRNA-221/-222 knockdown increased gefitinib sensitivity in resistant and sensitive gefitinib cell lines indicating that these miRNAs are important modulators of TKI resistance. Results from the same paper also show that MET overexpression controls gefitinib resistance through activation of the AKT/ERKs pathway, mediated at least in part by the miRNA-103 and -203 downregulation since an induced expression of these miRNAs increases Calu-1 cells gefitinib sensitivity. Additionally, Dicer knockdown reduced gefitinib resistance and also migration and the expression of mesenchymal markers. Since miRNA-103 targets Dicer, these results may suggest that this miRNA could be involved in the EMT process through Dicer down-regulation. Ultimately, all these results were supported by *in vivo* studies since miRNA-103 and miRNA-203 overexpression or miRNA-221 and -30c knockdown resulted in tumor growth inhibition and increased sensitivity to gefitinib in nude mice after treatment [59].

miRNA-214 has also been described as a gefitinib resistance mediator [60]. After exposure to increasing concentrations of gefitinib, studies performed by Y-S Wang and coworkers in the resistant clone of a lung adenocarcinoma cell line, HCC827/GR, revealed an overexpression of miRNA-214. The upregulation of this miRNA leads to a PTEN down-regulation, which is involved in PI3K–AKT pathway [57]. PTEN protein dephosphorylates PI3K, that mediates activation of AKT, ultimately leading to an inactivation of this pathway [38]. So, miRNA-214 mediates gefitinib resistance in this model by activating PI3K/AKT pathway, which has been described to confer resistance to EGFR-TKI by overcoming the EGFR blocking in previous studies [61]. Finally, miRNA-214 knockdown led to gefitinib sensitivity in HCC827/GR [60].

Additionally, other studies performed by Wang *et al.* also revealed a gefitinib resistance in NSCLC cell lines and *in vivo* models, but this time mediated by Axl-altered miRNAs. Findings of the involvement of Axl kinase in acquired resistance to TKIs in this tumor model were prior to this article but Wang and his fellow workers proposed the involvement of the miRNA-374a and miRNA-548b in this resistance [62]. Analysis of the miRNA expression profile was performed in a generated gefitinib-resistant cell line, HCC827-Gef, in Calu1 cell line, which is resistant to TKI, and in tumor samples. Results revealed a relationship between Axl overexpression and the overexpression of miRNA-374a

and downregulation of miRNA-548b not only in the gefitinib-resistant cell lines but also in tumor samples. Knockdown of miRNA-374a and upregulation of miRNA-548b increased the sensitivity to gefitinib in gefitinib-resistant cell lines revealing their importance in this mechanism. Finally, results from the same authors also showed that miRNA-374a and miRNA-548b not only have a role in gefitinib sensitivity and gefitinib-induced apoptosis but also essential roles in cell cycle arrest, EMT, migration and tumorigenesis of gefitinib-resistant lung cancer cells *in vitro* and *in vivo* by targeting WNT5A and CCNB1, respectively [63].

Gastric cancer

Gastric cancer (GC) is the fourth most commonly diagnosed cancer and the second most common cause of cancer related death worldwide [39]. Results from a recent large-scale Phase III study demonstrated that trastuzumab combined with standard chemotherapy provided a significant survival advantaged compared with chemotherapy alone in advanced HER2-positive GC [64]. Even though trastuzumab can prolong the survival of patients with HER2-positive GC, most of them end up developing resistance, highlighting the importance in clarifying the mechanisms behind this event [65].

Eto and coworkers described that miRNA-21/PTEN pathway regulated the sensitivity of HER2-positive GC cell lines to trastuzumab through modulation of apoptosis. On one hand, they were able to demonstrate that overexpression of miRNA-21 not only downregulated PTEN expression but also increased AKT phosphorylation, however, not affecting HER2 expression. On the other hand, suppression of miRNA-21 increased PTEN expression and downregulated AKT phosphorylation, still not affecting HER2 expression. In addition, overexpression of miRNA-21 decreased GC cells sensitivity to trastuzumab by suppression of apoptosis; whereas suppression of miRNA-21 expression restored trastuzumab sensitivity of GC cells. These findings suggest that miRNA-21/PTEN pathway may be crucial to trastuzumab acquired resistance mechanism in GC [66].

Breast cancer

Breast cancer remains the most frequently diagnosed malignancy and the primary cause of cancer-related death in women globally [51]. HER2 overexpression occurs in 10–34% of invasive breast cancers [41]. HER2-positive breast cancers are associated with more aggressive tumor phenotypes and often acquired resistance to therapy [67,68]. Additionally, the downregulation of key miRNA processing enzymes, such as Drosha and Dicer, have been associated with the outcome, progression and recurrence of breast cancer. In fact, it was shown that Dicer is an independent predictor of recurrence in the HER2-positive subtype [40,42,69]. Ye and coworkers showed that in a HER2-positive breast cancer cell line, SK-BR-3, miRNA-221 knockdown led to a significant decrease of surviving cells in the presence of trastuzumab, while overexpression of the pre-miRNA in question led to the opposite result. Trastuzumab resistance in this tumor model seems to be mediated by tumor suppressor PTEN, since a miRNA-221 overexpression leads to a PTEN downregulation [43,70].

The activation of IGF1R, an alternative growth factor receptor, represents a common feature of trastuzumab-refractory cells [71]. However, the underlying mechanism remained unclear until very recently, when Xing-Ming and coworkers demonstrated that epigenetic silencing of miRNA-375 induces trastuzumab resistance in HER2-positive breast cancer by targeting IGF1R [72]. Their findings revealed that miRNA-375 targeted IGF1R and was downregulated in trastuzumab-resistant HER2-positive breast cancer cells. While overexpression of miRNA-375 restored trastuzumab sensitivity in cells, inhibition of miRNA-375 induced trastuzumab resistance in HER2-positive breast cancer cells. They also showed that regulation of miRNA-375 expression was epigenetic since inhibition of DNA methylation and histone deacetylation restored the expression of miRNA-375 in trastuzumab-resistant cells. Additionally, they found a negative correlation between the levels of miRNA-375 and IGF1R in breast cancer tissue samples. Lastly, epigenetic silencing of miRNA-375 causes IGF1R upregulation, which at least partially explains the mechanism of trastuzumab resistance in breast cancer cells [72].

A similar work was performed by Gong *et al.* which consisted in *in vitro* experiments and *in vivo* analysis of HER2-positive breast cancers. HER2-positive-trastuzumab-resistant cell lines were obtained by doing cell cultures in the presence of low-dose trastuzumab. miRNA analysis concluded that miRNA-21 was overexpressed in all resistant cell lines in comparison with parental ones. On one hand, knockdown of this miRNA resensitized the trastuzumab resistant breast cancer cells and its effects in proliferation and cell cycle. On the other hand, ectopic expression of miRNA-21 led to trastuzumab resistance in parental cell lines. Trastuzumab resistance mediated by miRNA-21 appears to be via tumor suppressor PTEN, since overexpression of this miRNA led to lower PTEN protein levels. Retrieving PTEN expression in resistant breast cancer cells restored trastuzumab activity, since this targeted therapy enhances PTEN phosphatase activity leading to AKT dephosphorylation [73]. *In vivo* studies were also performed and similar results were obtained leading to

the conclusion that miRNA-21 overexpression in tumor xenografts results in resistance to trastuzumab by inhibiting PTEN expression. Finally, the clinical significance of these findings was evaluated by examining primary breast cancers from patients receiving trastuzumab therapy. miRNA-21 expression in breast cancer cells was reversely correlated with PTEN expression, and in line with a miRNA-21 upregulation, PTEN expression was lower in trastuzumab-resistant tumors [44].

Also, recent studies performed by Corcoran *et al.* in breast cancer cell lines revealed an involvement of miRNA-630 in resistance to HER-targeting drugs such as lapatinib, neratinib and afatinib [74]. Lapatinib-resistant SKBR3 and HCC1954 cells and neratinib-resistant HCC1954 cells showed a decrease in intra and extracellular levels of the miRNA-630 when compared with the parental cell lines. Transfection of miRNA-630 mimic to resistant cell lines enhanced the

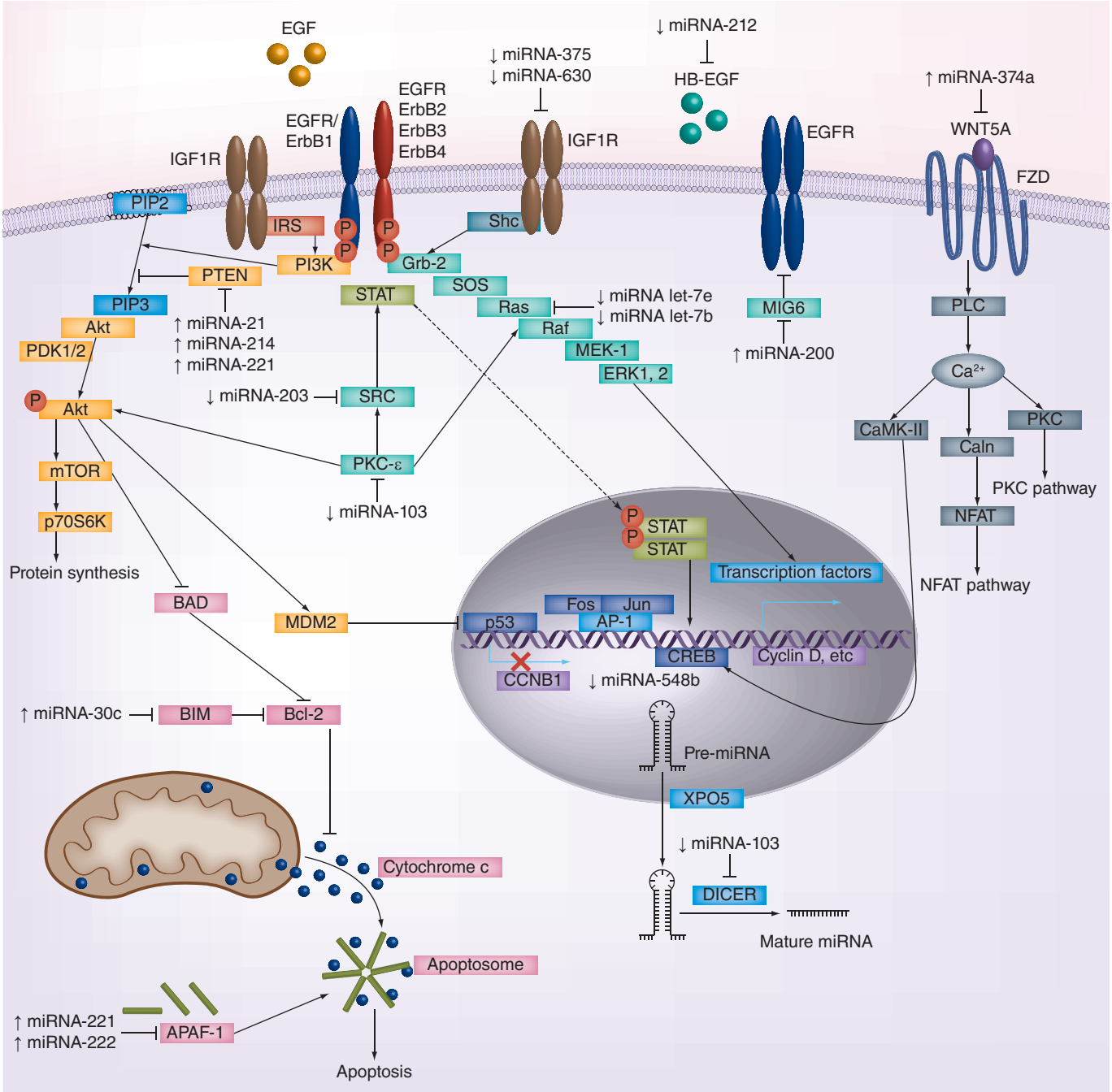

Figure 1. Schematic representation of the miRNAs involved in ErbB targeted therapy acquired resistance and their targets in the multiple cell signaling pathways.

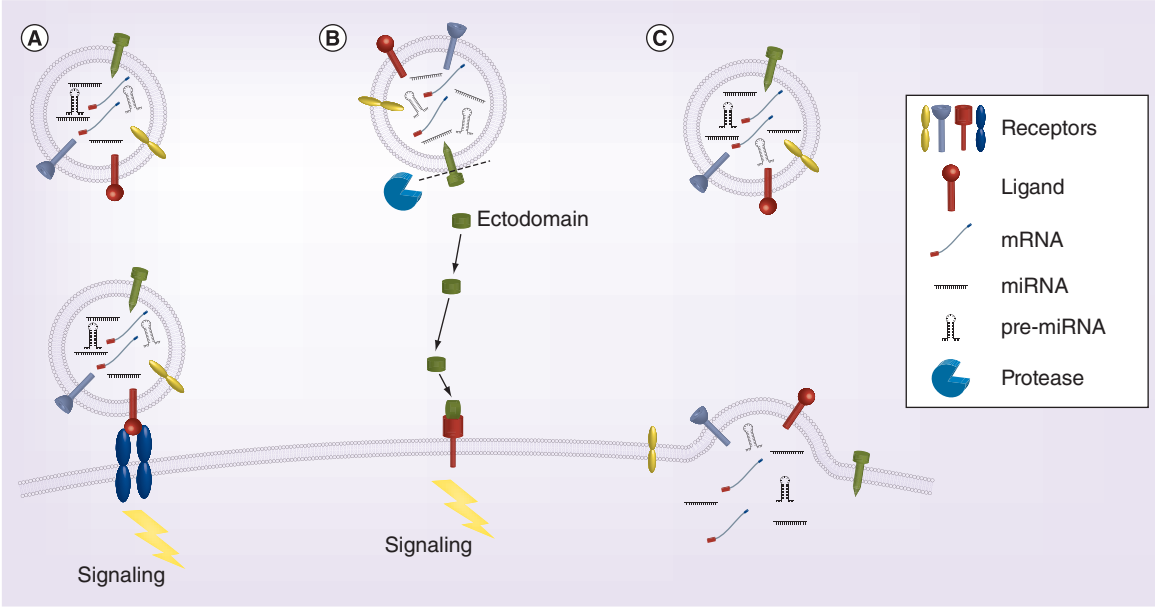

Figure 2. Possible mechanisms of exosome-target cell interaction. (A) Interaction between exosomal membrane proteins and target cell receptors leads to intracellular signaling activation – juxtacrine fashion; **(B)** Interaction between an ectodomain, formed after protease cleavage of exosomal membrane proteins, and target cell receptors also leads to signaling pathways activation; **(C)** Fusion of the exosome with the recipient cell membrane and content release to the intracellular space in a nonselective manner.
Adapted with permission from [75].

antiproliferative effects of all the drugs in study. The miRNA-630 dependent resistance mechanism seems to be related, not only with the increase of IGF1R levels, a target of miRNA-630, but also with the increase of EGFR and HER2 levels, targets of the drugs in study. The levels of the phosphorylated form of these proteins are also increased when miRNA-630 inhibition is performed in breast cancer cell lines. Finally, Corcoran *et al.* also proved that inhibition of the miRNA-630 in breast age-matched cancer cells was associated with increased motility, migration, invasion and resistance to anoikis [74].

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death, affecting men and women almost equally [75]. The use of monoclonal antibodies against EGFR, such as cetuximab and panitumumab, are a common pharmacologic approach in CRC treatment [76]. Besides the discovery that acquired *KRAS* mutations are a good predictive marker of cetuximab and panitumumab resistance in CRC, it is still unclear why certain patients respond to therapy and others do not [76,77].

Ragusa and coworkers made an expression profile of 667 miRNAs in two human colorectal cancer cell lines, one cetuximab sensitive (Caco-2) and other cetuximab resistant (HCT-116). They identified a group of miRNAs differentially expressed and tested them in CRC patients. miRNAs let-7b and let-7e were downregulated in HCT-116 after cetuximab treatment, in which signaling downstream of KRAS remains activated. Let-7 family members are known to target KRAS, so their downregulation could be a mechanism that contributes to cetuximab resistance. Additionally, miRNA-17* (a CRC marker) is up-regulated in the resistant-cell line and downregulated in the sensitive one, after cetuximab treatment [78]. Taken together, miRNA let-7b, let-7e and 17* could be considered as candidate molecular markers of cetuximab resistance [79].

Regarding all this results, we observe that, in some cases, different miRNAs are involved in different acquired resistance mechanisms for the same drug and in the same tumor model. miRNAs are not specific for a single mRNA, moreover mRNAs and consequently proteins are not regulated by only one miRNA [6]. Additionally, signaling pathways related with ErbB family overlap [26]. With this being said, it is more likely that an acquired resistance to a targeted therapy would be mediated by a network of miRNAs rather than a single one, targeting multiple steps of different pathways (Figure 1). However, since miRNAs play a major role in targeted therapy resistance, more specifically, resistance to ErbB family targeted therapies, we could consider them as therapeutic options. The use of miRNAs mimics or inhibitors, when drug resistance is due to an under-expression or overexpression, respectively, of the miRNA in question, should be consid-

ered as adjuvant therapy to drugs such as cetuximab, erlotinib, gefitinib and others.

The main issue regarding the use of miRNAs as therapeutic options is the specificity of the delivery *in vivo*. In other words, a direct delivery to the tumor, without affecting other body parts. Many studies have been made which led to huge improvements in this area and this introduces our next topic, exosomes.

Exosomes: a potential drug delivery model in EGFR-overexpressing tumors

Exosomes are membrane-bound vesicles of 40–100 nm in diameter present in almost all biological fluids [80]. They are released from most cell types, including cancer cells, into the extracellular space after fusion with the plasma membrane [81]. This type of extracellular membrane vesicles are enriched in cholesterol, sphingomyelin and ceramide as well as lipid raft associated proteins [81,82]. As a consequence of their origin, nearly all exosomes, independently of the cell type from which they originate, contain similar composition. However, the exosomal lumen, which is in part composed by mRNAs, miRNAs and other noncoding RNAs is determined by the cell type which produced the exosomes [83]. In the last years, many authors reported differences in miRNA content when comparing exosomes from normal individuals with cancer patients [84,85]. Since exosomes are released and are able to circulate in most biological fluids, they can interact with neighboring or distant cells and ultimately lead to the modulation of the recipient cells [86]. There are three main possible mechanisms of intracellular communication by exosomes. First, in a juxtacrine fashion, exosomal membrane proteins can interact with receptors in a target cell and activate different signaling pathways. Second, proteases in the extracellular space can cleave exosomal membrane proteins, leading to a cleaved fragment-target cell surface receptor interaction. This mechanism also activates intracellular signaling. Finally, exosomes can fuse with the target cell membrane and release their contents, such as mRNAs and miRNAs, which can alter gene expression and protein translation of the recipient cell (Figure 2) [81]. The ability of exosomes to interact and modulate target cells and also their high stability in circulation makes them good candidates to *in vivo* delivery of different molecules, such as miRNA mimics or inhibitors, which would allow us to surpass targeted therapies resistance mediated by miRNAs [87]. In fact, exosomes have already been used in *in vivo* models with different purposes and in a wide-range of diseases [88]. More specifically, in cancer Phase I clinical trials, exosomes are being used to, either increase innate and adaptive immune responses against the tumor, or deliver therapeutic agents in a cancer-specific way [88]. Related with the exosomes high stability and ability of travelling in biological fluids, a Phase I clinical trial is investigating the ability of plant exosomes to deliver curcumin to normal and colon cancer tissue, since previous studies demonstrated that curcumin has a strong inhibitory effect on the growth of colon cancer cell lines by mediating signal transduction [89,90].

Regarding the focus of our review article, more recently, Ohno *et al.* showed that exosomes could be used as drug delivery carriers in an EGFR-overexpressing cancer model. They used modified exosomes, with GE11 peptide in their membranes, to specifically deliver exosomal content to EGFR-expressing breast cancer cells. GE11 peptide binds to EGFR and is markedly less mitogenic than EGF. Also, in the same paper, efficient *in vivo* delivery of let-7a, a miRNA that functions as a tumor suppressor, was achieved by loading it to GE11^{+} exosomes and by injecting the modified exosomes intravenously in RAG2$^{-/-}$ mice, that were submitted, previously, to breast cancer cells transplantation [91]. This treatment suppressed tumor growth and no major organ damage was detected in the injected mice [92].

Although an exosome-based drug delivery model seems promising, many challenges still rise and have to be overcome. One of the main issues is the inexistence of a standard technique to isolate and purify exosomes. Usually, ultracentrifugation is used to obtain exosomes, however this technique has some limitations since it leads to low production yield and contamination with protein aggregates and cellular debris, which may affect the quality of these nanovesicles [93]. Another issue is the lack of biochemically well-characterized exosomes and the fact that the exosome protein content varies depending on the cells that produced them [83,93]. These facts could determine the safety and effectiveness of an exosome-based treatment since certain molecules, such as MHC class I or II, could trigger a host immune response and eliminate these vesicles. Finally, the method of drug loading to exosomes should be optimized since the efficiency is relatively low [93]. Once these issues are surpassed, exosomes could become a novel therapeutic approach not only in cancer but in many other diseases allowing a more specific and potent drug effect.

Conclusion

miRNAs are an important epigenetic mechanism of acquired resistance to targeted therapies by cancer cells. Despite great findings lately, this subject still needs further research in order to completely understand the mechanisms underlying acquired resistance of different drugs in a wider range of tumor types.

Executive summary

miRNAs

- miRNAs are a class of noncoding RNAs that control gene expression by either degrading or blocking translation of mRNAs.
- A miRNA is not specific for a certain mRNA, it can regulate up to 100 different mRNAs and is also described that more than 10,000 mRNAs seem to be regulated by miRNAs.
- Changes in miRNA levels may have an effect in treatment response, since targeted therapies are used for specific proteins and signal pathways related with biological processes, which are regulated by miRNAs.

Cancer treatment

- Chemotherapy and radiotherapy target, but not exclusively, cancer cells, causing several side-effects. Target therapy emerged in order to minimize those side-effects.
- Despite all the latest developments and improvements in cancer therapy, resistance to treatment still exists.
- Acquired chemotherapy drug resistance can be modulated by genetic and/or epigenetic factors. In fact, recent data demonstrate that certain miRNAs activity might be altered in order to achieve chemotherapy resistance.
- The ErbB/HER receptors family regulate apoptosis, cell cycle progression, cytoskeletal rearrangement and many other biological processes. Mutations or increased expression of ErbB family members occur in several malignancies, thus different ErbB/HER targeted therapies have been developed against this family of receptors.
- miRNAs may be a way not only to unveil resistance mechanisms but also, if used as a treatment option, to overcome targeted therapy limitations.

miRNAs & targeted therapy resistance

- One potential mechanism of acquired resistance to cetuximab in head and neck squamous cell carcinoma involves the increased expression of HB-EGF due to a decrease in miRNA-212 expression. HB-EGF promotes cetuximab resistance since it activates receptor kinases other than EGFR, like HER3 and MET.
- High expression levels of miRNA-21 are associated with gefitinib resistance in non-small-cell lung cancer (NSCLC) cell lines and also with trastuzumab resistance in HER2 positive gastric and breast cancer cell lines. The upregulation of this miRNA leads to a PTEN downregulation, which is involved in PI3K-AKT pathway. PTEN protein dephosphorylates PI3K that mediates activation of AKT, ultimately leading to AKT pathway inactivation.
- miRNA-214 and miRNA-221 overexpression are associated with gefitinib resistance in a lung adenocarcinoma cell line and trastuzumab resistance in a breast cancer cell line, respectively. Since PTEN is a target of these miRNAs, the resistance mechanism is also based in a PI3K/AKT pathway activation.
- In different lung cancer cell lines it has been demonstrated that TGF-β–miRNA200–MIG6 pathway coordinates the EMT-associated kinase switch that induces resistance to erlotinib.
- Garofalo and coworkers demonstrated that changes in MET and EGFR-related miRNAs levels, more specifically, miRNA-30c, miRNA-103, miRNA-203, miRNA-221 and miRNA-222 levels, had a significant role in gefitinib resistance on NSCLC cell lines and *in vivo* models.
- The overexpression of miRNA-374a and the downregulation of miRNA-548b have been correlated with Axl kinase overexpression that is involved in acquired resistance to gefitinib in NSCLC.
- miRNA-375 downregulation by epigenetic silencing causes IGF1R upregulation, which leads to trastuzumab resistance in HER2-positive breast cancer.
- In breast cancer cell lines, miRNA-630 downregulation is associated with resistance to HER-targeting drugs such as lapatinib, neratinib and afatinib. The miRNA-630 dependent resistance mechanism seems to be related, not only with the increase of IGF1R levels, a target of miRNA-630, but also with the increase of EGFR and HER2 levels, targets of the drugs in study.
- Let-7 family members are known to target KRAS, so let-7b and let7-e downregulation could be a mechanism that contributes to cetuximab resistance in colorectal cancer.

Exosomes: a potential drug delivery model in EGFR-overexpressing tumors

- Exosomes are membrane-bound vesicles released from most cell types, including cancer cells.
- The exosomal lumen, which is in part composed by mRNAs, miRNAs and other noncoding RNAs is determined by the cell type which produced the exosomes.
- Exosomes can interact with surroundings or distant cells and ultimately lead to the modulation of the recipient cells, since they are able to circulate in most biological fluids.
- The ability of exosomes to interact and modulate target cells and also their high stability in circulation makes them good candidates to *in vivo* delivery of different molecules, such as miRNA mimics or inhibitors.
- In Phase I clinical trials, exosomes are being used to, either increase innate and adaptive immune responses against the tumor, or deliver therapeutic agents in a cancer-specific way.

Ultimately, these findings would allow a better understanding of tumor biology and therapy response, enforcing the development of a treatment even more personalized and efficient. In this review, we have highlighted miRNAs and their role in the development of acquired resistance to ErbB family targeted therapies in different cancer models.

Future perspective

In the future, miRNAs could be used as biomarkers for treatment response, through an expression profile, and also as therapeutic options. Modified exosomes carrying antitumor miRNAs are a promising drug delivery model that can revolutionize today's approach of cancer treatment.

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